THE ROLE OF MICROBIAL PROCESSES IN SOIL PHOSPHORUS DYNAMICS

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Historically, several different mechanisms have been proposed to explain soil P transformations, which are strongly influenced by hydrology. Areas in the landscape prone to saturate and produce runoff may, therefore, become important P sources, as well as some strategies for controlling non point source (NPS) P pollution, such as Vegetated Filter Strips (VFSs). Recently, the discovery of numerous microbial processes potentially significant for P transformations has challenged the traditional abiotic perspective of P cycling. Their role has been investigated in this research. Particularly, the potential effects of three processes in P release were evaluated: the decay of soil microbial biomass, the activity of Polyphosphate Accumulating Organisms (PAOs) and dissimilatory iron (Fe) reduction. The experimental approach was divided in two parts. For the first one, undisturbed soil cores from a VFS receiving silage leachate were maintained under flooding and draining cycles with acetate and glucose as carbon (C) sources, followed by an aerobic P enrichment period in order to promote polyphosphate (polyP) storage by PAOs. P release during flooding was dominated by organic forms, suggesting the contribution of the decay of soil microbial biomass. No polyP was found in the soils containing acetate following P enrichment, as revealed by liquid state ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR). This indicates that PAOs were indeed inactive, since their presence in the cores was confirmed

later in the second part of this research. Fe reduction was observed in the cores containing glucose, supporting the microbial nature of this process, although no concomitant inorganic P (P_i) release occurred. In the second part of this research, the presence of known groups of PAOs and dissimilatory Fe reducers, i.e. Accumulibacter and *Geobacteraceae*, respectively, was determined in the field site and the soil cores from the first part of this research using Polymerase Chain Reaction (PCR) based techniques. Clone libraries were constructed for Accumulibacter*, Geobacteraceae* and total bacteria. They were also quantified using quantitative PCR (qPCR). The resulting spatial distribution patterns of Accumulibacter and *Geobacteraceae* in the study site constitutes important evidence of their potential role in soil P dynamics.

BIOGRAPHICAL SKETCH

Maria Vicenta Valdivia Santibáñez was born in Coquimbo, Chile, in 1973. She graduated in 1996 with a Bachelor in Aquaculture Sciences at Universidad Católica de Norte in Chile, and in 1998 she received the professional title of Aquacultural Engineer from the same university. Back in her country, she worked at the Department of Marine Resources of Fundación Chile, a non-profit, private organization, where she developed a deep interest in environmental sciences and the impact of human activities in aquatic ecosystems. In 2005 she graduated with a M.Sc. in Biological and Environmental Engineering at Cornell University, and continued a Ph.D. program in the same department. Currently, she is the Director of the Aquacultural Engineering Program at Universidad Andrés Bello in Chile.

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First of all, I would like to thank my advisor, Dr. Todd Walter and the members of my special committee, Dr. Ruth Richardson, Dr. Murray McBride and Dr. Dan Buckley for their valuable guidance during the development of this research. I would also like to thank Larry Goehring, as well as Joshua Faulkner and Wei Zhang, for their feedback about the field site; to Francisco Flores-Lopez and Josephine Archibald for their assistance in field sampling; to Dr. Shree Giri for his advice about chemical analyses; to Dr. Ivan Kerestes and Dr. Tony Condo for their help with the analysis of soil extracts with liquid state ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR); and finally, to the Buckley lab for their invaluable help with the microbial ecology analyses as well as with many other aspects of this research. I would like to thank in special to Dr. Michael Walter, for his enlightenment during all these years. I would also like to thank my family and friends, for their love and support.

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CHAPTER 1

Understanding Soil Phosphorus Dynamics: Biogeochemistry

Abstract

Non Point Sources (NPS) of phosphorus (P) are a leading contributor to freshwater eutrophication, which is a persistent and acute global water quality problem. Many of the strategies used to minimize such pollution are not always effective, since their design is often based on processes involved in soil P dynamics that are yet not well enough understood. Vegetative Filter Strips (VFSs) are one commonly employed practice. However, they are prone to saturation, and P release is a common phenomenon in flooded soils. Some of the mechanisms proposed to explain P fluxes under flooding conditions include P release from soil microbial biomass due to the decay induced by osmotic shock and shifts in the microbial community; the potential role of facultative bacteria capable of luxury aerobic P uptake and anaerobic P release, i.e. Polyphosphate Accumulating Organisms (PAOs); and the release of P bound to iron (Fe) oxides as the result of Fe reduction. In this research, the significance of such mechanisms of P release in flooded soils from a VFS receiving silage leachate were investigated by maintaining undisturbed soil cores under flooding and draining cycles with acetate and glucose as carbon (C) sources, followed by an aerobic P enrichment period in order to promote intracellular polyphosphate (polyP) storage by PAOs. P release during flooding was dominated by organic forms, probably derived from the decay of soil microbial biomass due to an initial osmotic shock and a subsequent shift in the

soil microbial community. Fe reduction was observed in the cores containing glucose, supporting the microbial nature of this process, although no concomitant inorganic P (P_i) release occurred. This indicates either the absence of P bound to Fe oxides in these soils resulting in no P release at all, or that P was indeed released but rapidly mineralized by developing facultative and anaerobic microbial groups in the soil cores. No polyP was found in the soils containing acetate following P enrichment, as revealed by liquid state ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR). This indicates that PAOs were indeed inactive, since their presence in the cores using molecular techniques was confirmed later, as reported in Chapter 2. The results of this study suggest that microbial processes might be indeed significant in soil P cycling, and the implications of these findings are further discussed in this paper.

1. Introduction

Freshwater eutrophication is a persistent and acute global water quality problem. Non Point Sources (NPS) of phosphorus (P) are the leading cause of eutrophication in rivers, lakes and reservoirs, and over the years substantial efforts have been made to mitigate their effects on water quality. The effectiveness of many of the strategies implemented, however, is quite limited because many of the processes involved in soil P dynamics are not yet well enough understood to develop successful control practices.

Evidence indicates that soil P cycling at a landscape scale is largely driven by hydrology. In a sub-humid boreal forest, Devito et al. (2000) found that the

main sources of total P to lakes in the same region were hydrologically active, near surface flows from well organized ephemeral draws and wetlands. These observations were confirmed later by Macrae et al. (2005) in the same area, who found markedly high concentrations of total dissolved P (TDP) in areas at the toes of hillslopes compared to other places, regardless of land management. They also found that in such areas high TDP concentrations coincided with low total P in the soil, thus exhibiting a high ratio of TDP to extractable soil P, i.e. the soil-water partitioning coefficient. Similar patterns were found in a filter strip receiving nutrient-rich water from a milkhouse, where the locations with the highest soluble reactive P (SRP), i.e. orthophosphate or dissolved inorganic P (DP_i), in the water generally exhibited the lowest soil-bound P (Murray, 2001). This study also reported reduced SRP spring retention, which agrees with trends for TDP found in wetlands (Kovacic et al., 2000). Furthermore, some authors have reported enhanced SRP net exports during summer in buffer strips (Liljaniemi et al., 2003) and wetlands (Tanner et al., 2005). The implications of these findings are noteworthy, since areas in the landscape that are conceptually considered P sinks may, under particular environmental conditions, become P sources. This appears to be particularly true for areas likely to have saturated soils.

Some areas in the landscape are more prone than others to saturate and produce runoff. Such areas exhibit an enhanced hydrologic sensitivity relative to non runoff generating areas that needs to be considered in controlling non point source pollution because of the potentially rapid transport of nutrients and pollutants between the soils in these hydrologically sensitive areas (HSAs) and surface water bodies (Walter et al., 2000). HSAs are dominated by

Variable Source Area (VSA) hydrology, a watershed process whereby saturated areas are the primary sources of runoff. This name was originally attributed to Hewlett and Hibbert (1967), but the concept was further developed by Dunne (1970), Dunne and Black (1970), Hewlett and Nutter (1970) and Dunne et al. (1975).

While we refer to areas especially prone to soil saturation as HSAs, areas with disproportionately high reaction rates relative to their surroundings have been defined by McClain et al. (2003) as biogeochemical hot-spots. These areas commonly occur at the boundary or ecotone between two features in a landscape, with water playing a very important role enhancing biogeochemical activity. According to this concept, HSAs might coincide with biogeochemical hot-spots, particularly for P transformations which, according to evidence might potentially dynamic in such areas. Certainly, flooding of soils is known to enhance P release. The mechanisms controlling such fluxes are diverse and depend on the hydrological conditions of the soils prior to flooding. It is possible, then, to recognize three different situations: flooding after long term drying, intermittent flooding, and long term flooding.

After long term drying, the flooding of soils generally enhances P fluxes, which are often dominated by organic forms derived mostly from soil microbial biomass (Turner and Haygarth, 2001). Rapid rehydration after prolonged drought can kill between 17% and 58% of soil microbes by inducing cell rupture due to osmotic shock (Salema et al., 1982; Kieft et al., 1987). The physical stress induced by soil drying can also disrupt organic matter coatings on clay and mineral surfaces (Bartlett and James, 1980), thus further

contributing to the observed organic P fluxes when soils are rehydrated (Turner et al., 2002).

When intermittent flooding occurs, soils are often able to retain certain degree of moisture. Thus, soil microbes, rather than being exposed to an osmotic shock, are in fact selected based on their ability to withstand the anaerobic environment, which gradually evolves as oxygen is depleted due to biotic consumption. This might result in a P flux dominated, as in the previous case, by organic forms derived from the decay of strictly aerobic microbes, as the result of a microbial community shift. In addition, soils exposed to intermittent flooding generally display fluctuating water tables that can induce alternating redox potentials, conditions that may promote a phenomenon that has long been observed in sediments. The role of facultative bacteria capable of luxury aerobic P uptake and anaerobic P release has been proposed as an important redox-dependent mechanism for P exchange between sediments and water (Fleischer, 1983, 1986; Gächter et al., 1988; Davelaar, 1993; Gächter and Meyer, 1993; Goedkoop and Pettersson, 2000; Khoshmanesh et al., 2002; Hupfer et al., 1995, 2004, 2007; Maassen et al., 2005). These so called Polyphosphate Accumulating Organisms (PAOs) have been extensively studied as part of the Enhanced Biological Phosphorus Removal (EBPR) process in laboratory scale sequencing batch reactors (SBRs), pilot-scale systems and full-scale wastewater treatment plants (WWTPs) (see review by McMahon et al., 2007). This process alternates aerobic and anaerobic steps that result in fluctuating redox potentials in order to promote the removal of inorganic P (P_i) from wastewater. The consensus of the metabolic models for EBPR, as described by Garcia et al (2006), is that P_i is taken up from

wastewater by PAOs and converted into polyphosphate (polyP) during the aerobic period. During the anaerobic period, these PAOs break the phosphodiester bonds of the stored polyP to provide an energy source for taking up volatile fatty acids (VFAs), mostly acetate and propionate, and store them as polyhydroxyalkanoates (PHA) such as polyhydroxybutyrate (PHB). Evidence of biogenic polyP in sediments using ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR) supports the ecological role of these microorganisms in P cycling (Hupfer et al., 1995, 2004; Carman et al., 2000; Khoshmanesh et al., 2002; Reitzel et al., 2006, 2007). Their identity, however, remains speculative.

In soils under long-term flooding, iron (Fe) reduction is considered the dominant source of P release (Willett, 1989; Shahandeh et al., 2003). The classic Einsele-Mortimer model (Einsele, 1936, 1938; Mortimer, 1941, 1942) emphasize the abiotic view of P dynamics by coupling it to the redox-dependent reduction of Fe oxides in response to fluctuating oxygen levels in pore water. The presence of amorphous and hydrous oxides in the soil, which provide surface hydroxyl groups for anion chemisorption, has then long been considered one the most important factors affecting P retention, and the reduction of these oxides the dominant mechanism of P release in flooded soils (Willett, 1989; Shahandeh et al., 2003). While the effects of Mn and Al oxides on P retention are somehow similar to those of Fe, the importance of Mn in P release is limited to initial stages of soil reduction and to the presence of large amount of reactive Mn oxides (Shanahdeh et al., 2003), i.e. in soils with Fe : Mn content ratios less than five. Al reduction, on the other hand, is not redox sensitive, and thus Al oxides play a role only in terms of P retention

by constituting a long term P sink (Kopacek et al., 2005). In recent years, the discovery of bacteria capable of dissimilatory Fe(III) reduction has challenged the purely chemical view of this process. There is a wide diversity of bacteria and archaea with this metabolic capability (Lovley, 2000a,b). Nevertheless, environments where Fe reduction is an important electron acceptor process are dominated by the *Geobacteraceae* family (Anderson et al., 2003; Holmes et al., 2002; Roling et al., 2001; Snoeyenbos-West et al., 2000; Stein et al., 2001). *Geobacteraceae* belongs to the δ subclass of proteobacteria and comprises five genera, i.e. *Geobacter*, *Desulfuromonas*, *Desulfuromusa*, *Pelobacter* and *Malonomonas*, which have mostly been isolated from anoxic sedimentary environments (Holmes et al., 2004). All *Geobacteraceae*, with the exception of *Pelobacter*, are capable of complete oxidization of multicarbon compounds such acetate to carbon dioxide using Fe as electron acceptor (Lovley, 2000b).

Precipitation of P minerals is another important process controlling soil P concentrations. Variscite ($AlPO_4 \cdot 2H_2O$) is the main regulator of phosphate solubility in many acid mineral soils, while in non acid soils P solubility is controlled primarily by the formation of calcium (Ca) phosphate minerals (McBride, 1994). The importance of precipitation in soil P fluxes, however, varies in different soils according to mineralogical characteristics, making extremely difficult to make generalizations, particularly based on the hydrological status of the soils.

Upon the depletion of oxygen in flooded soils, anaerobic degradation of organic matter takes place with a succession of thermodynamically favored

reactions starting with the reduction of nitrate (NO_3^-), followed by Mn(IV), Fe(III), sulfate (SO_4^{2-}) and carbon dioxide (CO_2), in this order as each electron acceptor is successively depleted. Acetate has been identified as the main intermediate in anaerobic mineralization of organic carbon (C) in many aquatic ecosystems (Lovley and Klug, 1982; Rothfuss and Conrad, 1993), playing also an important role in upland environments, such as prairie and forest soils, where anaerobic conditions are largely restricted to microsites in soil aggregates (Küsel and Drake, 1994; Wagner et al., 1996). In rice field soils, acetate is the dominant fatty acid, often accumulating at high concentrations within two weeks after flooding (Inubushi et al., 1984; Klüber and Conrad, 1998; Krylova et al., 1997; Sugimoto and Wada, 1993). Glucose apparently served as an important precursor of acetate in such environments, accounting for 54 to 81% of the acetate produced (Chidtaisong et al., 1999). Moreover, it is the most abundant monosaccharide in field rice soils as well as in freshwater and marine ecosystems (King and Klug, 1982; Wicks et al., 1991.Jørgensen and Jensen, 1994; Boschker et al., 1995; Dulov et al., 1995; Hanisch et al., 1996). The availability of acetate in soil microsites as well as in subsurface ecosystems might then contribute to the predominance of particular bacterial groups, like PAOs and Fe reducers, which can be potentially important in P cycling in such environments.

Traditional strategies for controlling NPS of P have generally ignored the biological complexity of environmental P cycling, with the possible exception of plant uptake. Vegetated Filter Strips (VFSs) are one of the most commonly used strategies for reducing P NPS pollution and epitomize this point. The underlying logic behind their design is that soluble P will be sorbed by the VFS

soil matrix and retained there. VFSs are classified within the category of what are known as buffer areas, which have the purpose of intercepting, retaining and transforming nutrients and pollutants (Dorioz et al., 2006). The efficiency of VFSs in minimizing pollution has, nevertheless, been the subject of controversy with respect to P. Under normal conditions there is no biogeochemical transformation capable of reducing the amount of P stored in soils. Thus, P accumulates until soils reach their maximum retention capacity, when retention and release rates come into equilibrium, and can no longer store additional P. The situation is further complicated in the case of VFSs because they are intentionally positioned to capture surface waters and, as such, are highly susceptible to flooding. Such systems must then receive special attention, given that their main purpose of reducing P pollution might paradoxically result in further exacerbating this problem.

In this research, three mechanisms of P release in flooded soils from a VFS receiving silage leachate were investigated: organic P release due to microbial decay triggered by flooding, P release associated with PAO activity, and P release associated with Fe reduction. Undisturbed VSF soil cores were maintained under flooding and draining cycles, in the presence of acetate and glucose as C sources. Upon the completion of the flooding cycles, a P enrichment period was performed in all the cores to promote polyP formation by PAOs. Flooding water biogeochemistry was monitored daily and soil samples from the cores containing acetate were analyzed with liquid state ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR) to determine de presence of polyP. The objectives of this study were **i)** to determine the main mechanisms of P release during soil flooding; **ii)** to obtain evidence of the

potential effect of PAOs in soil P fluxes by promoting fluctuating redox conditions in the presence of acetate followed by an aerobic P enrichment period; and **iii)** to determine the occurrence of Fe reduction with concomitant P release.

2. Methods

2.1. Field site description

The study site is a VFS located on a large Concentrated Animal Feeding Operation (CAFO) dairy farm in Freeville, Tompkins County, in central New York. It is approximately 4 years old, and designed for treating leachate from horizontal bunker silos using a slow rate infiltration process. The system is divided into two cells, each 65.5 m long by 36.6 m wide, and receiving the leachate from four pipes located at the entrance of the cells (Fig. 1.1). The site has 5% north-south slopes on a Langford channery silt-loam, with a fragipan at approximately 60 cm deep. Vegetation was originally seeded as follows: reed canarygrass (11 Kg/ha), redtop (3 Kg/ha), and tall fescue (22 Kg/ha).

The chemistry of the soils in this study site has been previously characterized (L. Goehring, pers. comm.). These soils have 1.49% C and total P, Fe, Mn and Al contents of 802, 27,207, 608 and 21,447 mg/Kg, respectively, as determined by microwave digestion with nitric acid (HNO_3) followed by Induced Couple Plasma (ICP) analysis.

2.2. Soil cores extraction

Six undisturbed soil cores, on average 3.9 Kg (mass under field conditions) and 10 cm deep, were extracted from the study site in May 2007. For the extraction, PVC cylinders of 15 cm diameter and 20 cm deep were gently pounded into the soil. The soil surrounding the PVC core was then hand excavated and the cores were carefully removed. The bottom of each core was covered with a plastic layer, leaving a 5 cm drainage hole at the center. The gaps between the soil core and the PCV tubing were sealed by injecting high density polyurethane foam.

Figure 1.1. Field site with the location of the VFS cells (outlined in black) and distribution pipes (in red). The arrow shows the flow of silo bunker runoff to the distribution pipes.

2.3. Experimental design

A diagram of the experimental design is shown in Fig. 1.2. The six soil cores were placed in buckets and flooded with 13 L of tap water, leaving a head of about 10 cm over the soil surface. They were flooded for a period of 10 days followed by a 5-day draining period. This process was repeated until completing three flooding cycles. While draining, the surface of all cores was carefully checked to make sure the soils remained moist. The monitoring of the cores during the first flooding cycle allowed us to establish a time span based on the rate of the observed biogeochemical reactions; specifically, after 10 days, DO levels were near zero for all cores and the redox potentials were nearly constant.

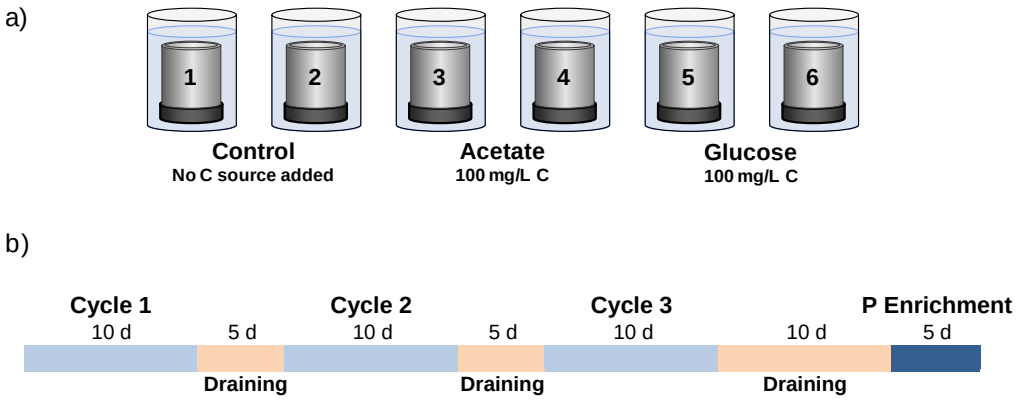

Figure 1.2. Diagram of the a) soil cores, and b) the experimental design for flooding cycles.

Soil cores designated as 1 and 2 were maintained as controls and flooded only with tap water, while the remaining four cores were supplemented with a C source at a concentration of 100 mg/L of C. Acetate (as CH_3COONa), was

added to cores 3 and 4, and glucose ($C_6H_{12}O_6$) to 5 and 6. The purpose of the alternating flooding and draining was to create fluctuating redox conditions in order to promote the development of PAOs. The anaerobic environment during flooding was also expected to promote Fe reduction with the concomitant release of P.

Upon completion of the three flooding cycles, there was a draining period of 10 days in order to remove excess of pore water. It was followed by an aerobic P enrichment period of 5 days, time span that was selected in order to avoid the formation of anaerobic soil microsites due to flooding. The six soil cores were placed in buckets containing 16 L of tap water with a concentration of 100 mg/L of P (as KH_2PO_4) and air supplied at saturation by air pumps through a diffuser. The cores were placed over a 5 cm platform which allowed water to freely circulate below the cores, which made the additional 3 L of water necessary to maintain a head of about 10 cm over the soil surface as in the flooding cycles. No C sources were added to the cores during the P enrichment period. The purpose of this step was to promote phosphate uptake and polyP accumulation, if PAOs were indeed present in the soil cores.

2.4. Samples and data collection

Values of pH, dissolved oxygen and redox potential were registered daily at the water column. Water samples for chemical analyses were collected with the same frequency. Redox potential was corrected by adding 200 mV to measured voltages, as required when using an Ag/AgCl reference electrode. Water temperature was also registered daily, remaining within 22-30°C during

the experiment. Water samples were stored at 4°C until analysis. Soil samples from the cores surface were taken at the beginning of the experiment and at the end of each flooding cycle and the P enrichment period, and stored at -20°C until analysis.

2.5. Chemical analyses

Water samples were filtered at 0.45 µm and analyzed by Inductively Coupled Plasma (ICP) for total phosphorus (TP), as well as for total iron (Fe), manganese (Mn), calcium (Ca) and aluminum (Al). Al concentrations were undetectable in all the samples analyzed. Inorganic phosphorus (P_i), as soluble reactive phosphorus (SRP), was analyzed by Flow Injection Analysis (FIA) using an OI Analytical FS2000® analyzer. The fraction of organic phosphorus (P_o) was estimated by subtracting P_i from TP values. Additionally, concentrations of acetate (CH_3COO^-), nitrate (NO_3^-) and sulfate (SO_4^{-3}) were analyzed using a Dionex IC-2000® Ion Chromatograph. Glucose in samples from cores 5 and 6 was analyzed by High Pressure Liquid Chromatography (HPLC). Only trace levels were detected, suggesting a rapid consumption within the first 24 h. These results are, therefore, not shown. Water samples taken from each soil core at the end of each flooding cycle were analyzed for dissolved organic carbon (DOC) by High Temperature (850°C) Platinum Catalyst Combustion using a Shimadzu 5000® analyzer. Although the water volume used during the P enrichment period (16 L) was 23% larger than the volume used for the flooding cycles (13 L), all concentrations are reported as they were measured, without any volume correction. The tap water used for flooding the cores contained TP, P_i and P_o, as well NO_3^- and SO_4^{-3}.

Soil samples corresponding to soil cores 3 and 4 collected at the end of the P enrichment period were analyzed by liquid state ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR), following Cade-Menun et al. (2005).

2.6. Statistical Analyses

Simple statistical correlations were performed between duplicates for all the available data, in order to corroborate the reproducibility of the observed trends. The Coefficient of Determination (r^2) was calculated in each case, and reported in Appendix I.

3. Results

3.1. Water chemistry during flooding cycles

TP concentrations increased over time in all the soil cores and were generally lower in the cores containing a C source than the controls (Fig. 1.3). These trends were observed in all the cycles. For P_i, the concentrations in cores with a C source remained almost undetectable in all the flooding cycles and were constant for all the columns. Thus, the increasing TP concentrations were primarily due to increases in P_o.

Ca concentrations increased over time in all the cores, with higher values in the ones with glucose (Fig. 1.4). The cores containing acetate displayed a behavior similar to the control cores in all the flooding cycles. The increase in Ca concentrations was less pronounced in cycles 2 and 3 than in cycle 1.

pH values were neutral to alkaline, relatively stable over time and similar in all the soil cores during cycle 1 (Fig. 1.5). During cycles 2 and 3, however, pH in the cores containing glucose was consistently lower, reaching a minimum at 96 - 144 h and stabilizing later. DO concentrations dropped during the first 24 h in all six soil cores, particularly in those containing a C source. Redox potential decreased rapidly and reached a minimum within the first 48 h, slightly increasing and stabilizing later. Compared to the control cores, the redox potential was generally lower for the cores containing glucose in all the cycles, and intermediate for the cores containing acetate. In soil cores 3 and 4, where acetate was added as a C source, concentrations decreased steadily over time, with only a fraction remaining at the end of each flooding cycle. Acetate was also measured in the rest of the cores. It remained undetectable in the control cores, but it appeared after 24 h in the ones containing glucose, increasing over time just to disappear by the end of each flooding cycle.

For all the flooding cycles, NO_3^- concentrations markedly decreased during the first 24 h for the soil cores containing a C source, particularly those with acetate, and remained low for the rest of each cycle (Fig. 1.6). In the control columns NO_3^- was relatively stable overall. Mn concentrations increased over time for all the flooding cycles, particularly the first two. They were comparatively low for the control cores, intermediate for the cores with acetate, and high for the two cores with glucose, particularly core 6. Fe concentrations were in general lower than those for Mn, with peaks appearing about the same time than Mn peaks in the cores containing glucose, being in this case higher in core 5 instead of core 6. During cycle 1, SO_4^{2-} concentrations increased over time in the control cores, while remaining

constant for the rest of the cores. During cycle 2, a similar trend was observed for the control cores, while the cores with a C source displayed remarkably lower SO_4^{2-} concentrations, particularly the cores with glucose. This pattern was even more evident during cycle 3.

3.2. Water chemistry during the aerobic P enrichment period

In general, pH increased over time, with slightly lower values for the cores containing glucose (Fig. 1.7). DO concentrations remained relatively stable, with a minor drop by the end of the enrichment period. Redox potential was very stable over time. TP and P_i concentrations were very similar, and decreased steadily over time in all soil cores, although this trend was less pronounced in the control cores than in the cores with a C source. Many of the estimated P_o concentrations were negative values, which is not unexpected when the fraction of organic P forms is small since this is indeed an operationally defined fraction (data not shown). Ca remained relatively constant over time, with lower concentrations in the cores with a C source. NO_3^- concentrations varied with no obvious trend over time in all the cores, with slightly higher values at the end of the experiment than at the beginning. Mn concentrations remain undetectable (data not shown). Fe was released from all the soil cores and reached a peak in most of the cores at 48 h, stabilizing later at lower concentrations. SO_4^{2-} concentrations increased steadily over time in all soil cores, particularly in those containing a C source.

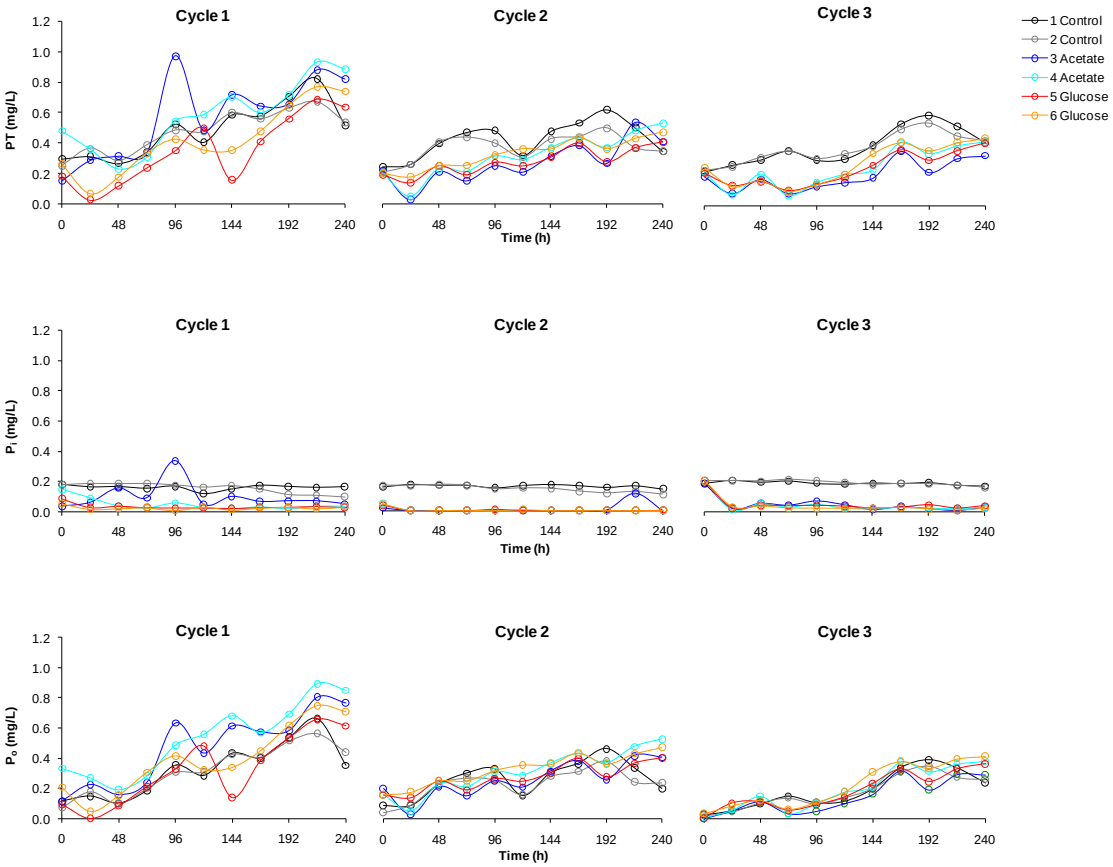

Figure 1.3. Total (TP), inorganic (P_i) and organic (P_o) phosphorus concentrations (mg/L) during the flooding cycles.

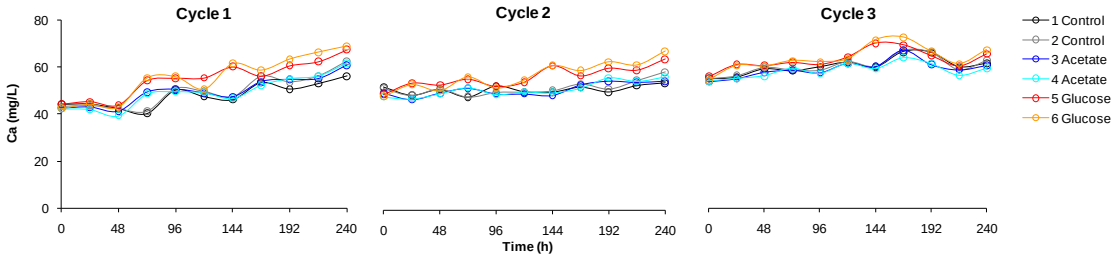

Figure 1.4. Calcium (Ca) concentrations (mg/L) during the flooding cycles.

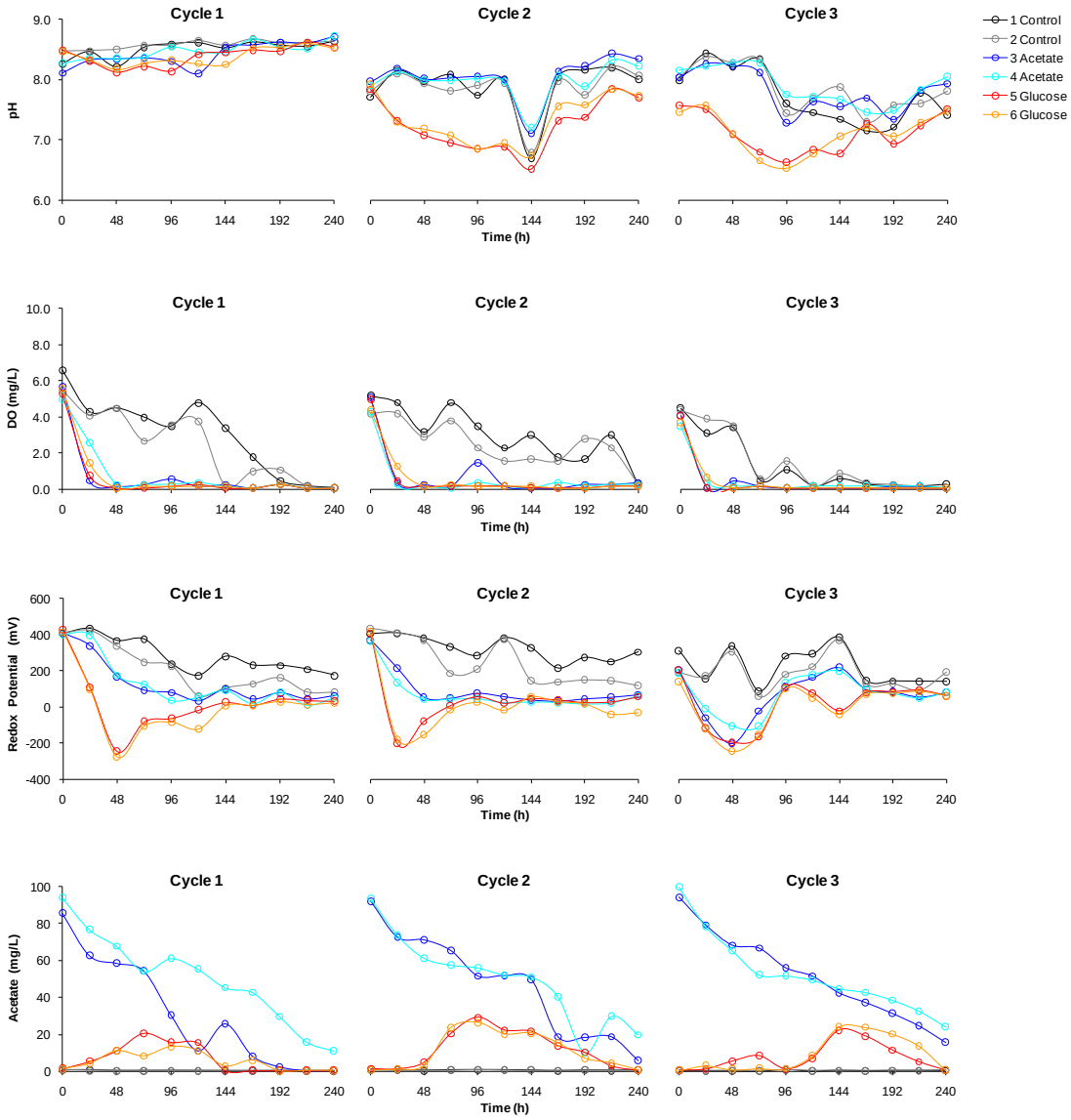

Figure 1.5. pH, DO (mg/L), redox potential (mV) and acetate concentrations (mg/L) for soil cores during the flooding cycles.

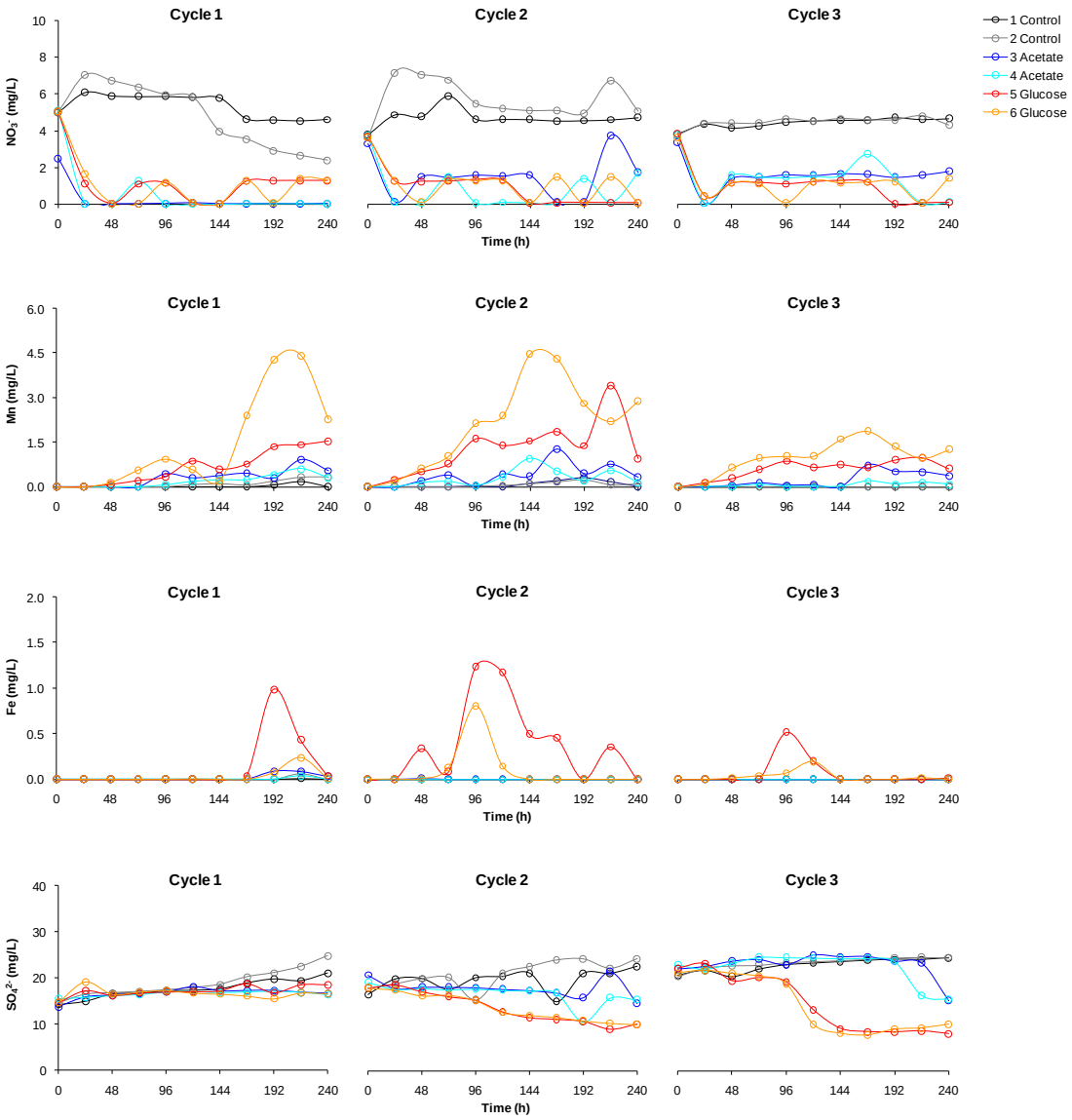

Figure 1.6. Nitrate (NO_3^-), manganese (Mn), iron (Fe) and sulfate (SO_4^{2-}) concentrations (mg/L) during the flooding cycles.

3.2. Water chemistry during the aerobic P enrichment period

In general, pH increased over time, with slightly lower values for the cores containing glucose (Fig. 1.7). DO concentrations remained relatively stable, with a minor drop by the end of the enrichment period. Redox potential was very stable over time. TP and P_i concentrations were very similar, and decreased steadily over time in all soil cores, although this trend was less pronounced in the control cores than in the cores with a C source. Many of the estimated P_o concentrations were negative values, which is not unexpected when the fraction of organic P forms is small since this is indeed an operationally defined fraction (data not shown). Ca remained relatively constant over time, with lower concentrations in the cores with a C source. NO_3^- concentrations varied with no obvious trend over time in all the cores, with slightly higher values at the end of the experiment than at the beginning. Mn concentrations remain undetectable (data not shown). Fe was released from all the soil cores and reached a peak in most of the cores at 48 h, stabilizing later at lower concentrations. SO_4^{2-} concentrations increased steadily over time in all soil cores, particularly in those containing a C source.

3.3. Dissolved Organic Carbon (DOC)

DOC at the end of each flooding cycle was comparatively lower in the control cores, intermediate in the ones with glucose and higher for the cores with acetate, particularly for cycle 3 (Fig. 1.8). Interestingly, DOC at the end of the aerobic P enrichment period was higher than DOC after each flooding cycle in the control cores and the ones with glucose.

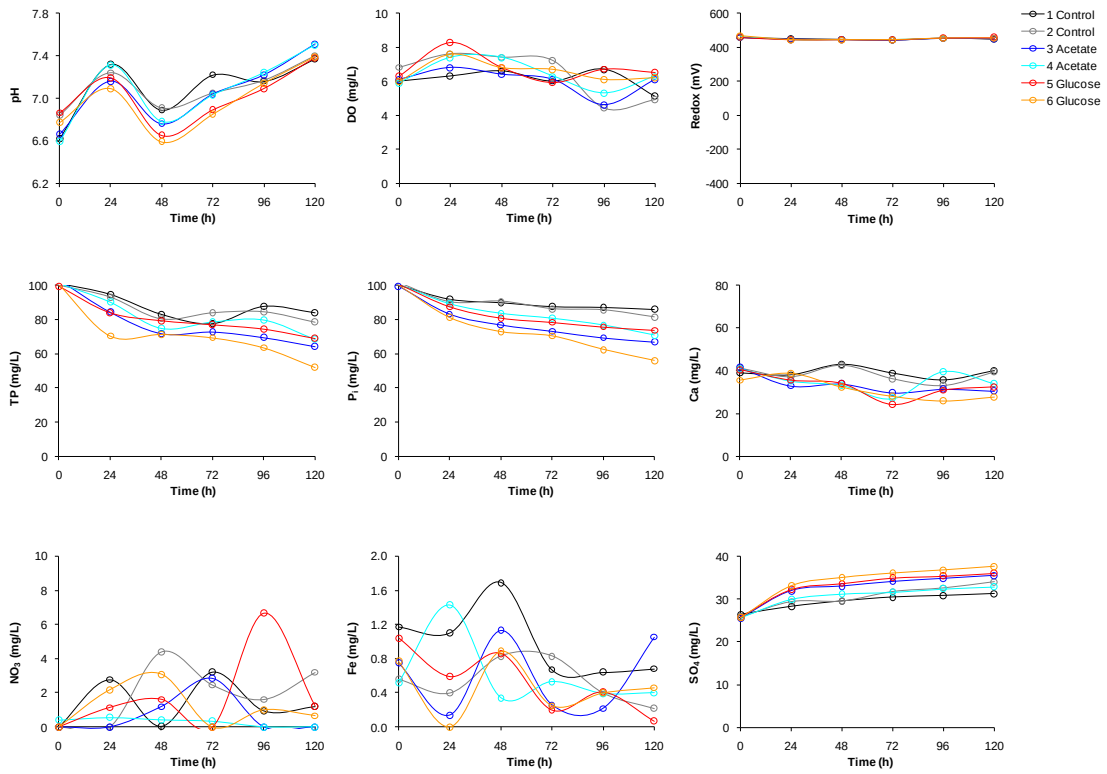

Figure 1.7. pH, DO (mg/L), redox potential (mV), total phosphorus (TP), inorganic phosphorus (P_i), calcium (Ca) and sulfate (SO_4^{2-}) concentrations (mg/L) in soil cores during the P enrichment period.

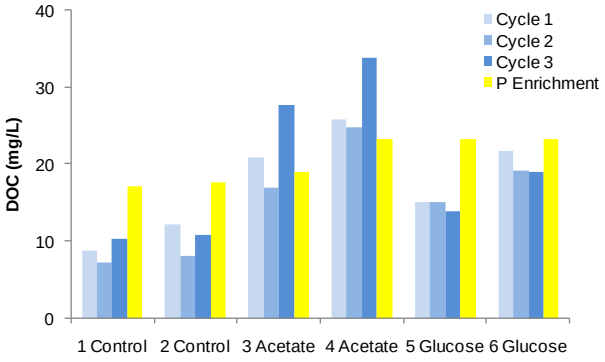

Figure 1.8. Dissolved Organic Carbon (DOC) concentrations (mg/L).

3.4. ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR)

Results were almost identical for soil cores 3 and 4. The ^{31}P-NMR analysis revealed that soil P was present primarily as ortho-P (peak at 6.4 ppm), with a large fraction of ortho-P monoesters (peaks in the region of 4 to 6 ppm) (Fig. 1.9). Peaks at -0.5 and -1 ppm indicate the presence of a small fraction of ortho-P diesters, i.e. phospholipids and DNA, respectively. The presence of polyP with terminal P groups was revealed by a very small peak at -4 ppm, although long chain polyP at -20 ppm was not detected.

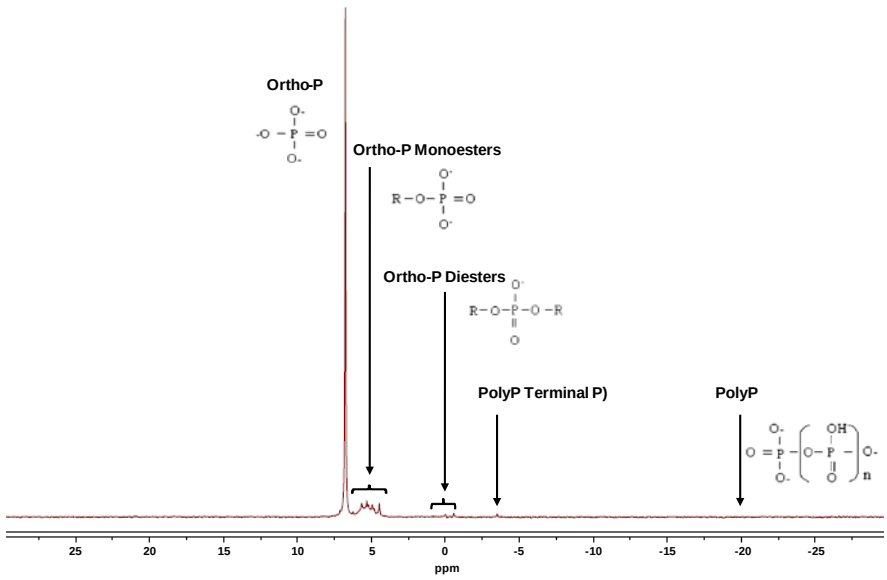

Figure 1.9. ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR) spectrum in soil core 4.

4. Discussion

4.1. Phosphorus

In general, there was a sustained release of phosphorus as TP over time during flooding in all the soil cores (Fig. 1.3). These results are in agreement with the general theory that P is released from flooded soils as a result of an environment that becomes increasingly anaerobic with the concomitant drop in the redox potential (Shahandeh et al., 2003). Nevertheless, TP release was slightly larger in the control cores, suggesting an underlying mechanism for P retention in the presence of C additions. P_i concentrations showed this trend even more evidently, since while concentrations in the control cores remained constant over time, P_i in the cores with acetate and glucose drop almost immediately after flooding, remaining almost undetectable for the rest of the time for all the flooding cycles. Thus, the increased observed in TP was primarily due to the P_o, which as remarkably similar for all the cores.

P fluxes dominated by an organic fraction have been attributed primarily to the release of P from soil microbial biomass. After long term drying, rapid rehydration can kill soil microbes by inducing cell rupture due to osmotic shock (Salema et al., 1982; Kieft et al., 1987). Such effect, however, has been observed within a time span of 48 h (Turner and Haygarth, 2001), and does not appear to explain a sustained P_o release from flooded soils over a longer period of time, as observed in these experiments. Nevertheless, since the soil cores in this experiment were flooded in batch conditions, the contribution of such P_o fluxes during initial stages of flooding cannot be dismissed.

Soil microbes are sometimes able to resist osmotic stress (Griffiths et al., 2003). The costs associated with tolerating this stress are different for different organisms, depending on their inherent resistance and acclimation abilities (Gordon et al, 2008), and thus they can lead to a shift in the composition of the microbial community (e.g. Fierer et al., 2003). It is, therefore, likely than the observed sustained P_o release in this experiment is due to the decay of strictly aerobic microbes due to the increasingly anaerobic environment gradually promoted by oxygen depletion during flooding. A shift in the community composition also implies the development of microbial groups better adapted to the new prevailing environmental conditions. The disruption of organic matter coatings on clay and mineral surfaces due to flooding could also have contributed to organic Po fluxes, although most likely during early flooding and primarily in cycle 1, since the soil cores were in general drier at beginning of the experiment.

The undetectable P_i concentrations in the cores with a C source are indeed counterintuitive to the mechanisms proposed to explain P release in flooded soils, i.e. the role of PAOs and Fe reducers. They might be the result, however, of P_i uptake as structural element by developing facultative and anaerobic microbial groups. It might be even possible that P_i fluxes are underestimated based on the observed concentrations, which are only the net end result. The lack of P_i release during Fe release agrees with this theory. It is, nevertheless, impossible with the available evidence to determine whether P_i was indeed released along with Fe and rapidly consumed, or if it was not released at all. This latter implies the lack of P bound to amorphous Fe oxides in the study site. The extent of the effect of Fe reduction on P dynamics

requires, therefore, further research in order to evaluate the alternative mechanisms proposed.

An alternative explanation for these results is that anaerobic conditions could have lead to high carbon dioxide (CO_2) pressure in soil water. This generates high bicarbonate in soil solution, causing the dissolved Ca to rise, which was indeed observed. The presence of acetate and other organic acid anions generated under anaerobic conditions might have further contributed to increase Ca solubility. The higher dissolved Ca could have then have induced phosphate precipitation as Ca phosphate (McBride, pers. comm.).

The absence of polyP in the soils containing acetate suggests two possibilities. Either PAOs are absent in the soils of the study site, or they are actually present but not actively uptaking P and synthesizing polyP for intracellular storage. Further studies conducted with polymerase chain reaction (PCR) based molecular techniques revealed the presence of a known PAO in the soil cores, results that are described in detail in Chapter 2. This means that for some reason PAOs in the soil cores amended with acetate are indeed inactive. Interestingly, many of the microbially mediated biogeochemical reactions observed in this study occurred in the soil cores amended with glucose, including Fe reduction which is known to be promoted by acetate, suggesting that glycolysis during flooding and the gradual appearance of acetate as an intermediate compound may have an effect on thermodynamically favored processes following NO_3^- reduction. Therefore, ^{31}P-NMR analysis of soils from the cores amended with glucose could have provided an additional piece of information for PAOs activity.

Precipitation of P minerals may also contribute P availability, particularly phosphate. While precipitation of variscite ($AlPO_4 \cdot 2H_2O$) is limited to acid soils, the formation of Ca phosphate could be important in the soils used in this experiment. Nevertheless, the sustained increase of Ca in all the soil cores during flooding (Fig. 1.4) suggests that the extent of Ca phosphate precipitation is likely irrelevant in this study. Furthermore, the presence of reduced Mn has shown to inhibit the formation of thermodynamically stable Ca phosphates (Cao et al., 2007). This could in fact explain the slightly higher Ca concentrations in the cores containing glucose during flooding, which were the only ones displaying Mn reduction. Thus, the small differences between the cores with glucose and the rest of the cores could further support the idea that precipitation of Ca phosphate in this experiment is probably not significant.

During the aerobic P enrichment period, TP and Pi concentrations steadily diminish over time (Fig. 1.7). Interestingly, this decrease seems to be more pronounced in the soil cores that were amended with C during flooding, maybe as the result of microbial uptake. This might constitute a further line of evidence to support the development of a higher microbial biomass in the presence of a C source. Nevertheless, the contribution of other processes like sorption, cannot be dismissed. Precipitation, on the other hand, it is still unlikely, since besides the reasons previously provided, Ca concentrations remain relatively constant during this period.

4.2. Calcium

Soils are able to resist pH changes though several buffering mechanisms. Under conditions of low pH, soils exchange the added acid cations, i.e. H^+, by base cations, i.e. Ca^{2+}, which are then leached (McBride, 1994). This explains the steady increase in Ca concentrations observed during flooding in all the soil cores, particularly in those containing glucose, which exhibited the lowest pH. Mn reduction, as explained above, might account for the slightly higher Ca concentrations in the presence of glucose. During the aerobic P enrichment period, Ca concentrations remain relatively constant as well as the pH.

4.3. Biogeochemical response of flooded soils

Flooding induced a reducing environment in all of the soil cores, particularly in those containing a C source, as indicated by a sharp drop in DO concentrations and redox potentials (Fig. 1.5). This was particularly noticeable for the cores containing glucose, which reached the lowest pH values compared to the rest of the cores. The higher pH values in the presence of acetate can be explained by the production of hydroxide ions due to acetate hydrolysis. Similar trends were obtained by Wang et al. (2007) who incubated lake sediments with acetate and glucose.

DO was rapidly consumed within the first 24 h after flooding. It coincided with a drop in the moderate NO_3^- levels (Fig. 1.6), which was an indication of NO_3^- reduction. Mn release was then observed, reaching a peak later on in the cores containing glucose, particularly core 6, and surprisingly when there were

still some levels of NO_3^- in the water. Fe release was delayed with respect to Mn, and interestingly only observed in the presence of glucose (Fig. 1.6). SO_4^{2-} concentrations in the water decreased progressively over time in all the flooding cycles, primarily in the cores containing glucose. Assuming the decrease in NO_3^- concentrations is due to reduction, the progression of the observed apparent reductions agree with the sequence of biogeochemical reactions expected after flooding. There is no thermodynamic reason, however, why a reaction of less favorable energy should not also proceed (McCarty, 1972), which can explain the apparent overlap of processes, e.g. the occurrence of Mn reduction in the presence of NO_3^-, as well as Fe reduction in the presence of Mn.

The importance of acetate as an intermediate in anaerobic mineralization of organic carbon has been already stated, with glucose, apparently, serving as an important precursor. Such observations are confirmed by the results of this study, since acetate appeared in the soil cores containing glucose during all the flooding cycles, but it was absent in the control cores (Fig. 1.5). This is an indicator of a significant role of fermentative microorganisms as well as glycolysis in these soils during flooding. As mentioned before, many of the observed biogeochemical reactions in this study occurred in the soil cores amended with glucose, suggesting that acetate may have an important effect on promoting particular biogeochemical processes not only based on its availability but also in terms of the time at which it becomes available in accordance with the reducing status of the soils. Microbial populations commonly display a lag phase, which corresponds to an acclimation stage when they remain almost inactive until favorable thermodynamics conditions

gradually appear. Thus, a process such as NO_3^- reduction, which occurs almost immediately after flooding, might have been stimulated by acetate in the cores amended with this C source at an early stage of flooding (Fig. 1.6). Therefore, NO_3^- reducers could have been favored in terms of biomass earlier than Fe reducers, which at that point of soil reduction were likely still in the lag phase, competitive advantage that was prolonged until the end of each flooding cycle. Certainly, in all the flooding cycles, particularly cycle 1, NO_3^- concentrations dropped more sharply within the first 24 h in the cores containing acetate than in the ones with glucose, supporting this observation.

4.4. Nitrate

In anoxic environments, two pathways of dissimilatory NO_3^- reduction have been identified: denitrification, by which NO_3^- is reduced to nitrite (NO_2^-) and then to dinitrogen gases (N_2O and N_2), and dissimilatory nitrate/nitrite reduction to ammonia reduction of nitrate and/or nitrite (Brunet and Garcia-Gil, 1996). Both pathways respond to different C sources. Thus, while glucose has proven to increase denitrification rates by stimulating both synthesis and activity of denitrifying enzymes as well as supporting the biomass of denitrifiers, acetate has shown to stimulate nitrate reduction to ammonium (Wang et al., 2007). In the present study, NO_3^- reduction was virtually absent in the control cores (Fig. 1.6). In contrast, it was indeed observed in the cores with a C source, with a drop of the NO_3^- concentrations within the first 24 h that was more evident in the presence of acetate, suggesting its consumption by NO_3^- reducers, as it has been previously observed (Achtnich et al., 1995). During the P enrichment period, NO_3^- was released from all the soil cores,

which can be attributed to nitrification of NH_4-N under aerobic conditions (Zia et al., 2001).

4.5. Manganese and iron

Glucose had a major effect in both Mn and Fe reduction, as evidenced by their release from the soil cores. The redox transformations of these metals are relatively easy to monitor since their reduced forms are soluble, except for the fraction bound to solid materials (Nealson and Saffarini, 1994). Although the reduction of both metals can occur simultaneously, the reduction of Mn is energetically favored in comparison to Fe, explaining why the release of Mn precedes that of Fe in all the flooding cycles (Fig. 1.6). It might also explain why the maximum concentrations of Mn were much higher than those of Fe, even though the total content of Fe in the soils in this particular study site is much larger than the content of Mn.

The rates of Mn and Fe reduction are difficult to estimate because of they are influenced by several factors. One of them is the nature of the available oxidized forms of Mn and Fe, i.e. amorphous oxides, in general, are more rapidly reduced than crystalline forms given their higher specific surface area (Langenhoff et al., 1997; Thamdrup, 2000). The relative fractions of different forms of Mn and Fe in the field site are unknown, making any estimation based on the available substrate unattainable. In addition, both metals can be readily reoxidized, and thus they are able to recycle as many as 100-300 times, as determined in offshore sediments (Canfield et al., 1993). The situation is even more complex, since sulfide interacts strongly with Mn and

Fe. It reacts with Fe(II) forming insoluble iron monosulfides (FeS), which can be further reduced by elemental sulfur to the more thermodynamically stable pyrite (FeS_2) at high sulfide concentrations (Howarth, 1979). In contrast, Mn sulfides are relatively soluble and precipitate only at very high concentrations (Nealson and Saffarini, 1994), being relatively stable in the presence of sulfide and often observed diffusing out of sulfide-rich zones in anoxic marine ecosystems (Nealson et al., 1991). Nevertheless, Mn(IV) as MnO_2 may rapidly react with sulfide, resulting in elemental sulfur and Mn(II) ((Nealson and Saffarini, 1994).

There is a diversity of bacteria capable of reducing Fe. They can use a number of different substrates as the preferential C source (Cummings et al., 2000; Finneran et al., 2003; Roh et al., 2002). Acetate serves a substrate for many Fe reducers, including *Geobacteraceae* which use it preferentially over other electron donors (Lovley, 2000b). Glucose has also been shown to serve this purpose when used as substrate directly (Lovley and Coates, 2000), although evidence suggests that glycolysis intermediates, and not glucose directly, might be responsible for transferring electrons to Fe(III) oxides (Lovley, 2000a). Glucose has been found to mainly degrade into acetate and CO_2 in paddy soils, with 54% to 81% of the acetate present as a glucose metabolite (Chidthaisong et al., 1999). Such evidence would explain the Fe reduction observed in the soil cores containing glucose, as explained before, which showed acetate formation after 48 h.

Interestingly, during the aerobic P enrichment period, Fe concentrations fluctuated with no apparent trend in all the soil cores within a range slightly

higher than the peak concentrations previously observed during flooding. Aeration of the cores during P enrichment could have had induced the release Fe reduced during flooding that was kept retained in pore water. In addition, Fe can be strongly bound to organic matter in comparison to Mn (Pakhomova et al., 2007). Thus, the release of organic matter in all the cores, as evidenced by the presence of DOC, could also explain the observed Fe release. In contrast, Mn concentrations remained undetectable during this period. Mn fluxes from sediments are in general correlated with Mn concentrations in pore water, except when ferromanganese nodules, crusts and manganese oxides are present in the sediment surface (Pakhomova et al., 2007). In such cases, dissolved Mn in porewater may be adsorbed and trapped in the surface layer of sediments, making Mn fluxes much lower than those predicted from its concentration gradient or fluxes may even be completely absent (Canfield et al., 1993). Thus, if reduced Mn was indeed present in pore water, it would not have been released if a layer of Mn oxides was formed at the cores surface during aerobic conditions.

4.6. Sulfate

SO_4^{2-} reducing bacteria (SRB) are a ubiquitous group of microorganisms capable of using sulfate as an electron acceptor during anaerobic respiration. They are phylogenetically diverse, including groups of gram-negative mesophilic, gram-positive spore forming, and thermophilic SRB (Castro et al., 2000). SRB play a major role in the cycling of organic matter in coastal marine environments (Jørgensen, 1982), where SO_4^{2-} concentrations are usually high. In contrast, SO_4^{2-} in soils is generally present at low concentrations (Nealson

and Saffarini, 1994). As with other electron accepting processes, the availability and quality of C sources has a strong influence on SO_4^{2-}. It has been demonstrated that the addition of acetate stimulates SRB (Chidthaisong et al., 1999). Although glucose has been show to have a similar effect, most known SRB do not metabolize glucose (Gibson, 1990; Widdel, 1988). Therefore, it is likely that the observed effects of glucose addition are due to the production of acetate as a degradation product. Interestingly, SO_4^{2-} reduction in the present study was noticed almost exclusively in the cores with glucose, which indeed showed acetate formation with peaks that coincided in time with the observed decrease in the SO_4^{2-} concentrations. The progression of SO_4^{2-} reduction over the flooding cycles suggests that the SRB population might have been in a lag phase prior to being metabolically capable of or thermodynamically favored for performing such process. During the aerobic P enrichment period, SO_4^{2-} concentrations increased steadily over time in all soil cores, particularly in those containing a C source. In aerobic conditions, a significant portion of the sulfide can be reoxidized to intermediate sulfur compounds and to SO_4^{2-} either chemically or biologically (Nielsen et al., 2006), explaining the observed increase in SO_4^{2-} concentrations.

4.7. General remarks

The amendment of soil cores with acetate and glucose as C sources promoted a more reducing environment in comparison with the control cores. Many of the observed biogeochemical reactions during flooding occurred in the cores containing additional C, which supports the theory that such processes are primarily microbially mediated. The differences between the amendment with

glucose and acetate as electron donors are most likely due to particular microbial groups being stimulated in terms of biomass at different stages of soil reduction, which obeys the sequence of thermodynamically favored reactions. Even when many reactions take place simultaneously, they are still energetically constrained. This observation, as well as the occurrence of a shift in the soil microbial community as the result of flooding is supported by the progression of the trends observed for many of the variables and chemicals analyzed. They are, indeed, gradually enhanced in the successive flooding cycles, suggesting an overall fitting of the system to the new environmental conditions. The high correlations between replicates ($r^2 > 0.7$, Appendix I) obtained for many of the observed trends confirm the reliability of the data.

5. Conclusions

The conclusions of this study are the following:

i) The release of P during flooding was dominated by organic forms, and probably derives from the decay of soil microbial biomass due to an initial osmotic shock and a subsequent shift in the soil microbial community.

ii) The absence of polyP in the soils containing acetate indicates that either PAOs are indeed absent in the soils of the study site, or that they are present but inactive and thus incapable of P uptake and polyP intracellular storage.

iii) No P release occurred along with Fe reduction, indicating either the absence of P bound to Fe oxides in these soils resulting in no P release at

all, or that P was indeed released but rapidly consumed by developing facultative and anaerobic microbial groups in the soil cores.

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CHAPTER 2

Understanding Soil Phosphorus Dynamics: Potential Microbial Controls

Abstract

A number of different mechanisms, both abiotic and biotic, have been proposed for explaining soil phosphorus (P) retention and release. In general, practices designed to control non point sources (NPS) of P are designed based on available knowledge about abiotic processes like soil P sorption. Nevertheless, a number of microbially mediated processes potentially significant for P cycling challenge this abiotic, purely chemical perspective. This study focuses on two biotic mechanisms: the potential role of facultative bacteria capable of luxury aerobic P uptake and anaerobic P release in the presence of acetate, i.e. Polyphosphate Accumulating Organisms (PAOs), and the release of P bound to iron (Fe) oxides as the result of the activity of dissimilatory Fe reducing bacteria. These processes may be particularly important in Hydrologically Sensitive Areas (HSAs), i.e. areas in the landscape prone to saturate and produce runoff. The purpose of this study was to determine the presence of known groups of PAOs and Fe reducers, i.e. *Candidatus* Accumulibacter phosphatis and *Geobacteraceae*, respectively, in the soils of a Vegetated Filter Strip (VFS) receiving silage leachate as evidence of their potential role in soil P dynamics. With the use of Polymerase Chain Reaction (PCR) based molecular techniques, clone libraries were constructed for three bacterial groups, Accumulibacter*, Geobacteraceae* and

total bacteria. They were quantified using quantitative PCR (qPCR) and their spatial distribution patterns obtained. The results indicate the presence of Accumulibacter and *Geobacteraceae* in the soils of the study site, constituting an important line of evidence of their potential roles in soil P dynamics. Accumulibacter was dispersed over the entire field site, with higher biomass within the VFS where moisture contents and, presumably, nutrient levels were higher than in the surrounding environment. In contrast, *Geobacteraceae* was clustered in distinctive areas that resembled sedimentary environments where this group has often been found. The biomass of total bacteria was high in the entire site, with evidence of bacterial transport with runoff. This is the first study demonstrating the presence of these two bacterial groups in a terrestrial environment where P is a potential water quality problem, along with revealing their spatial distribution patterns. The study of interactions between biotic processes performed by these and similar organisms and abiotic reactions may provide valuable insights to developing new, more effective strategies for controlling P loading on aquatic ecosystems.

1. Introduction

Soil phosphorus (P) cycling is largely driven by hydrology, and over the years many mechanisms have been proposed to explain the observed fluxes. The classic Einsele-Mortimer model (Einsele, 1936, 1938; Mortimer, 1941, 1942) emphasizes the abiotic view of P dynamics by coupling the redox-dependent reduction of iron (Fe) oxides in response to fluctuating oxygen levels in pore water to the release of bound inorganic forms of P. Thus, the presence of amorphous and hydrous oxides in the soil, which provide surface hydroxyl

groups for anion chemisorption, has long been considered one the most important factors affecting P retention, and the reduction of these oxides the dominant mechanism of P release in flooded soils (Willett, 1989; Shahandeh et al., 2003). While the effects of Mn and Al oxides on P retention are somehow similar, the importance of Mn in P release is limited to initial stages of soil reduction and to the presence of large amount of reactive Mn oxides (Shanahdeh et al., 2003), i.e. in soils with Fe : Mn content ratios less than five. Al reduction, on the other hand, is not redox sensitive, and thus Al oxides play a role only in terms of P retention by constituting a long a term P sink (Kopacek et al., 2005).

Soil P sorption, however, shows a more complex behavior than the implied by a simple adsorption model. Indeed, evidence suggests that P adsorption (surface complexation) and precipitation occur simultaneously (Khare et al., 2005). Adsorption appears to be the dominant mechanism regulating P solubility at low concentrations, whereas P mineral precipitation controls P solubility at high concentrations (Lindsay et al., 1989). The occurrence of surface precipitation can inhibit phosphate desorption by isolating some of the P from exchange solution (Li and Stanforth, 2000) and may, furthermore, increase the apparent sorption capacity of a mineral (van Riemsdijk and Lyklema, 1979; Laiti et al., 1996).

In recent years, the discovery of a number of microbially mediated processes potentially significant for P cycling has challenged the abiotic, purely chemical view predominant in many of the studies related to soil P transformations. For instance, it has been demonstrated that the flooding of soils after long term

drying enhances P fluxes dominated by organic forms, which are primarily derived from soil microbial biomass (Turner and Haygarth, 2001). Rapid rehydration in such conditions can kill between 17% and 58% of soil microbes by inducing cell rupture due to osmotic shock (Salema et al., 1982; Kieft et al., 1987). In conditions of intermittent flooding, soils are often able to retain moisture and rather than being exposed to an osmotic shock, soil microbes are in fact selected based on their ability to withstand the increasingly anaerobic environment gradually promoted by oxygen depletion due to biotic consumption under flooded conditions. This might result in a P flux dominated, as in the previous case, by organic forms as the result of the decay of strictly aerobic microbes.

The redox fluctuations taking place under a hydrologic flood pulse might promote a phenomenon that has been observed over the years in sediments. The role of facultative bacteria capable of luxury aerobic P uptake and anaerobic P release has been proposed as an important redox-dependent mechanism for P exchange between sediments and water (Fleischer, 1983, 1986; Gächter et al., 1988; Davelaar, 1993; Gächter and Meyer, 1993; Goedkoop and Pettersson, 2000; Khoshmanesh et al., 2002; Hupfer et al., 1995, 2004, 2007; Maassen et al., 2005). These so called Polyphosphate Accumulating Organisms (PAOs) have been extensively studied as part of the Enhanced Biological Phosphorus Removal (EBPR) process in laboratory scale sequencing batch reactors (SBRs), pilot-scale systems and full-scale wastewater treatment plants (WWTPs) (see review by McMahon et al., 2007). These microorganisms are capable of phosphate uptake and intracellular storage of polyphosphate (polyP) under aerobic conditions. When the

environment turns anoxic, the hydrolysis of stored polyP granules results in phosphate release with the concomitant uptake of organic acids, primarily acetate (Tchobanoglous et al., 2003). The identity of the microorganisms responsible of such process has long remained only speculative given the impossibility of isolating them in axenic culture. With the use of 16S rRNA based molecular techniques the first confirmed PAO to date, *Candidatus* Accumulibacter phosphatis (Hesselmann, 1999), was finally identified. Fluorescence in situ hybridization (FISH) has confirmed the dominance and role of this β proteobacterium, member of the family *Rodocyclaceae*, in EBPR systems, including lab-scale reactors, where it can be enriched to up 90% of total cells (Lu et al., 2006), pilot-scale systems and full-scale wastewater treatment plants (WWTP) (see review by McMahon et al., 2007). Besides FISH, Quantitative Polymerase Chain Reaction (qPCR) methods have been developed to measure the abundance and relative distributions of Accumulibacter clades in lab and full scale EBPR systems, targeting both 16S rRNA and polyP kinase 1 (ppk1) genes (He et al., 2007). The ppk1 enzyme is responsible of catalyzing the reversible reaction of polyP formation from ATP (Kornberg et al., 1999), thus being key in polyP metabolism. Recently, as part of the study of the global distribution of Accumulibacter in EBPR systems, the use of specific primers revealed its presence in freshwater and associated sediments, and also, although rarely, in soils (Kunin et al., 2008; Peterson et al., 2008). Positive samples were collected at varying distances from WWTP performing EBPR (Peterson et al., 2008), suggesting that Accumulibacter is dispersed in the environment from the open activated sludge aeration basins enriched in this organism via aerosols. Kunin et al. (2008) propose that Accumulibacter populations are environmentally distributed as sparse high-

density point sources, i.e. EBPR sludges, which are linked via widespread diffuse aquatic reservoirs, thus, constituting a metapopulation, i.e. a collection of contained populations connected by a small amount of gene flow (Hanski, 1999). The ecological role of this metapopulation in natural environments, however, remains unknown.

Another potentially important biotic link to soil P retention and release has emerged in recent decades with the increasing attention paid to the role of microorganisms capable of dissimilatory Fe(III) reduction. They are capable of strongly influencing P dynamics through releasing inorganic P compounds bound to Fe oxides during Fe reduction. There is a wide diversity of bacteria and archaea with this metabolic capability (Lovley, 2000a,b). Nevertheless, environments where Fe reduction is an important electron acceptor process are often dominated by the *Geobacteraceae* family (Anderson et al., 2003; Holmes et al., 2002; Roling et al., 2001; Snoeyenbos-West et al., 2000; Stein et al., 2001). *Geobacteraceae* belongs to the δ subclass of Proteobacteria and comprises five genera, i.e. *Geobacter*, *Desulfuromonas*, *Desulfuromusa*, *Pelobacter* and *Malonomonas*, which have mostly been isolated from anoxic sedimentary environments (Holmes et al., 2004b). All *Geobacteraceae*, with the exception of *Pelobacter*, are capable of complete oxidization of multicarbon compounds such as acetate to carbon dioxide using Fe as electron acceptor (Lovley, 2000b). This group has been previously quantified by targeting the gene that encodes the α subunit of the dinitrogenase protein, nifD, for fixing nitrogen (Holmes et al., 2004b, 2007). This capability seems to be widespread in this group, and likely allow these species to adapt and proliferate in nutrient limited environments.

The metabolic activity of Accumulibacter and *Geobacteraceae* has mostly been studied in EBPR systems and aquatic sediments, respectively. Very little related research has been focused on terrestrial ecosystems. Both soils and sediments often have a common origin, i.e. the weathering products of rocks and organic material, with water and time being the greatest distinguishing factors between them (Apitz, 2005). A particularity of sediments, however, is that they integrate inputs of nutrients and contaminants within a catchment or coastal region, often exhibiting enhanced rates of biogeochemical activity. Areas with disproportionately high reaction rates relative to the surrounding matrix have been defined by McClain et al. (2003) as biogeochemical hot-spots. These areas commonly occur at the boundary or ecotone between two features in a landscape, with water playing a very important role in these hot-spots.

Areas in the landscape especially prone to saturate exhibit an enhanced hydrologic sensitivity relative to the surrounding landscape, and hence often display an elevated biogeochemical activity, acting therefore as biogeochemical hot-spots. The rapid transport of nutrients and potential pollutants between the soils and surface water bodies by surface runoff from such areas need to be accounted for in control strategies P NPS pollution (Walter et al., 2000). These Hydrologically Sensitive Areas (HSAs) are dominated by Variable Source Area (VSA) hydrology, a watershed process whereby saturated areas are the primary sources of runoff. This concept was originally credited to Hewlett and Hibbert (1967), but the field experiments are often attributed to Dunne (1970) and Dunne and Black (1970). Later work by Hewlett and Nutter (1970) and Dunne et al. (1975) is also widely cited.

Vegetated Filter Strips (VFSs) are a special type of HSA because their propensity to saturate is largely due to their design. VFSs are one of the most commonly used strategies for reducing NPS of P. They are classified within the category of what are denominated buffer areas or buffer zones, which have the purpose of intercepting, retaining and transforming nutrients and pollutants (Dorioz et al., 2006). They are, hence, intentionally positioned to capture surface waters, which also makes them highly susceptible to soil saturation, flooding and runoff generation. They also receive a high load of different nutrients besides P, which often spreads the array of biogeochemical reactions taking place in these areas.

The purpose of this study was to determine and quantify, by using PCR-based molecular techniques, the presence of particular groups of PAOs and Fe reducers in the soils of a VFS receiving silage leachate, as evidence of their potential role in P dynamics. The objectives of this study were **i)** to determine the presence of Accumulibacter and *Geobacteraceae* in the field site using specific PCR primers; **ii)** to test the specificity of the primers and to determine the diversity of the bacteria present in the field site by constructing a clone library for Accumulibacter and *Geobacteraceae*, and for total bacteria, respectively; **iii)** to establish spatial distribution patterns for both groups and total bacteria based on qPCR; and **iv)** to quantify both groups as well as total bacteria by qPCR in undisturbed soil cores extracted from the study site and used in a previous study.

2. Methods

2.1. Study site and samples collection

The study site is a VFS located on a large Concentrated Animal Feeding Operation (CAFO) dairy farm in Freeville, Tompkins County, in central New York. It is approximately 4 years old, and designed for treating leachate from horizontal bunker silos using a slow rate infiltration process. The system is divided into two cells, each 65.5 m long by 36.6 m wide, and receiving the leachate from four pipes located at the entrance of the cells (Fig. 2.1). The site has 5% north-south slopes on a Langford channery silt-loam, with a fragipan at approximately 60 cm deep. Vegetation was originally seeded as follows: reed canarygrass (11 Kg/ha), redtop (3 Kg/ha), and tall fescue (22 Kg/ha). This site as no known history of soil amendments with activated sludge.

In May 2008, soil samples of the top 10 cm were collected from a total of 67 points, including both VFS cells and a perimeter surrounding the entire area. Geographic coordinates corresponding to each point were registered using a Garmin Map76CSx GPS. Soil samples were stored at -80°C until analysis. Leachate samples from the four pipes were also collected and briefly stored at 4°C until analysis.

In addition, six undisturbed soil cores, 10 cm deep and 15 cm in diameter, were extracted from the study site in May 2007 as part of a controlled flooding experiment in the lab. Soil samples were collected prior to initiating the experiment, and after each of the three flooding cycles as well as after the

aerobic P enrichment period. Samples were stored at -20^{o}C until analyzed. Experimental details are described in Chapter 1.

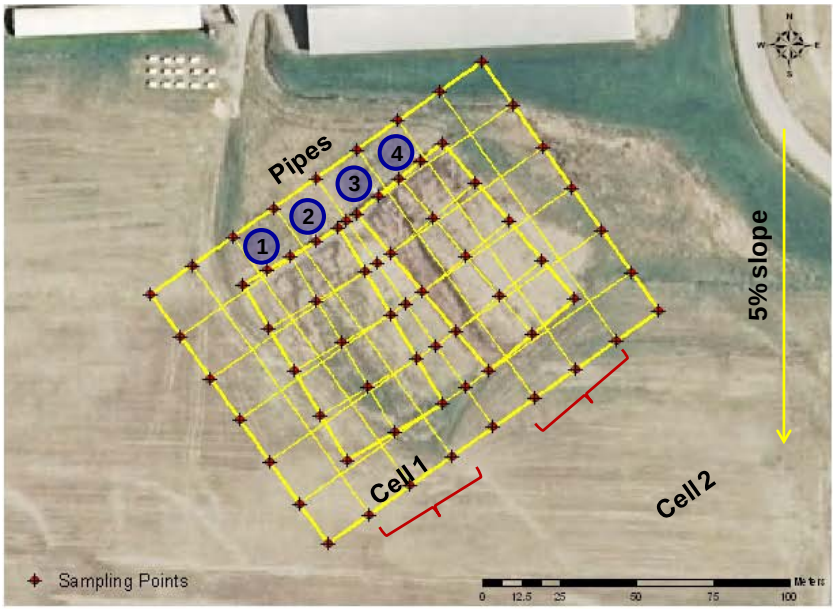

Figure 2.1. Field site with the location of the VFS cells, sampling points (in red) and distribution pipes (in blue).

2.2. DNA extraction and PCR analysis

Soil DNA was extracted from the samples using PowerSoil DNA Extraction Kit (MoBio), following the protocol proposed by the manufacturer. In addition to the samples taken at the field, samples from undisturbed soil cores previously extracted from this study site, which were maintained under flooding cycles followed by draining and a final aerobic P enrichment, were also processed for

DNA extraction. Using the same kit, DNA was also extracted from leachate samples, after obtaining a pellet by centrifuging 10 mL of leachate at 3,000 xg for 10 min. DNA concentration and purity in all samples were determined based on the absorbance at 260 / 280 nm.

Samples were screened with regular PCR using three primer pairs. The first pair targeted bacterial 16S rRNA genes. These primers have been previously used to quantify total bacteria (Yoshida et al., 2005; He et al., 2007). The second pair targeted Accumulibacter 16S rRNA, and the third one was specific for the α subunit of the dinitrogenase protein, nifD, in *Geobacteraceae* (Table 2.1). Amplicons were obtained in a MasterCycler Gradient (Eppendorf), using a 50 uL reaction containing 25 uL of Taq MasterMix (Qiagen), 0.5 uM of each forward and reverse primer, and 10 ng of DNA template. PCR programs for bacterial and Accumulibacter 16S rRNA genes consisted in an initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. For the *Geobacteraceae* nifD primers, the program started with an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 45 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 10 min. Annealing temperatures (Ta) for each primer set are in Table 2.1. PCR products were checked by agarose gel electrophoresis.

Table 2.1. Description of primers used.

Groups	Primer	Sequence	Amplicon size (bp)	Ta* (°C)	Reference
Accumulibacter	518f Acc-846r	CCAGCAGCCGCGGTAAT GTTAGCTACGGCACTAAAAGG	351	65	He et al. (2007)
Geobacteraceae	Geo-nifD-225f Geo-nifD-560r	ATCGGTGACGATATCAACGCC TAGTTCATGGAACGGTAGCAGT	335	60	Holmes et al. (2004b)
Total Bacteria	341f 534r	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	194	60	He et al. (2007)

*Ta: Annealing temperature (C)

2.3. Construction of clone libraries and phylogenetic analysis

A composite containing equal amounts of genomic DNA extracted from the 67 field samples was pooled in order to use it as a template for constructing clones libraries representative of the entire field site. Amplicons were obtained with regular PCR for three primer sets detailed in Table 2.1, according to the protocol previously described.

Clone libraries for each primer pair were constructed using the TOPO TA Cloning® Kit (Invitrogen), following the manufacturer's protocol. From each library, 30 positive clones were randomly picked, and checked by PCR. Amplified fragments were sent for sequencing to the Life Sciences Core Laboratories Center (CLC) at Cornell University.

The sequences for bacterial and Accumulibacter 16S rRNA genes were analyzed Classifier at the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/classifier/classifier.jsp), based on 80% confidence. This tool assigns 16S rRNA gene sequences to the new phylogenetically consistent higher-order bacterial taxonomy proposed by Garrity et al. (2007),

according to a naïve Bayesian rRNA classification algorithm (Wang et al., 2007). In the case of *Geobacteraceae* nifD genes, a sequence match was conducted in Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The sequences within each clone library, including related sequences that were included as a reference in each case, were aligned using CLUSTAL-W Multiple Alignment (Thompson et al., 1994) and phylograms were constructed using DNADIST version 3.5c, both interfaces within BioEdit Sequence Alignment Editor (Hall, 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylograms were edited with PhyloDraw Ver 0.8 (http://pearl.cs.pusan.ac.kr/phylodraw/).

In addition, the aligned sequences within each clone library were grouped into operational taxonomic units (OTUs) based on a 97% DNA sequence identity within each library. A distance matrix was computed using DNADIST version 3.5c, and the number of OTU within each library was calculated using the Average Neighbor Method in DOTUR (http://schloss.micro.umass.edu/software/dotur.html).

2.4. Quantitative PCR (qPCR)

Samples were screened with regular PCR for three primer sets detailed in Table 2.1, according to the protocol previously described. Positive controls for qPCR were obtained by extracting the plasmids from at least one positive clone from each library, using PureLink™ Quick Plasmid Miniprep (Invitrogen).

Concentrations of plasmid DNA were determined by absorbance at 260 nm, and the number of target copies estimated based on the molecular weight of each plasmid. A seven-point standard curve was constructed for each primer pair by 10-fold serial dilutions, starting with a maximum standard of 10^{10} copies per reaction for total bacteria, and 10^{8} copies for PAOs and *Geobacteraceae*.

Reactions were carried out in a 20 uL volume, containing 20 uL of SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen), 0.5 uM of each forward and reverse primer, and 5 ng of genomic DNA. Samples and standards were analyzed by triplicate in a 384-well plate, with a negative non-template control included. qPCR was conducted in a 7900 HT Sequence Detection System (Applied Biosystems). All qPCR programs consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing for 45 s, and extension at 72°C for 30 s.

qPCR results, expressed as number of copies / ng DNA, were transformed to number of cells / g of soil, based on the amount of soil DNA extracted from each sample and the number of copies of each target gene. The average number of 16S rRNA genes used for bacteria was 3.9, value obtained from the ribosomal RNA Operon Copy Number Database (rrnDB) (http://ribosome.mmg.msu.edu/rrndb/index.php) (Klappenbach et al., 2001; Lee et al., 2008). For Accumulibacter, this number was obtained from He et al. (2007), who estimated a value of 2. The value corresponding to *Geobacteraceae* nifD genes was 1, as determined by Holmes et al (2004a).

2.5. Spatial distribution of soil bacteria

Microbial biomass values, expressed as number of cells / g of soil, corresponding to the 67 field sampling points were processed using ArcGIS Suite 9.2 (ESRI). Briefly, data were interpolated using the Spline method, which estimates values using a mathematical function that minimizes overall surface curvature, resulting in a smooth surface that passes exactly through the input points. The method was applied using the tension option, which creates a surface with values more closely constrained by the sample data range.

3. Results

3.1. Phylogenetic analysis

The results obtained from RDP-Classifier for the 16S rRNA sequences of Accumulibacter and total bacteria are summarized in Tables 2.2 and 2.4. The classification in both cases was based on a confidence threshold of 80%. One of the bacterial 16S rRNA sequences (Total26) was not long enough to be analyzed in Classifier, probably due to the high number of unknown bases. Therefore only 29 sequences were reported. The results corresponding to the top matches from BLAST for the nifD sequences of *Geobacteraceae* are summarized in Table 2.3. All the matches were based on a maximum identity equal to or higher than 74%. For two of the sequences in this library (Geo3 and Geo4), no significant matches were found. Therefore, they were not included in the table.

Phylograms for the sequences of Accumulibacter, *Geobacteraceae* and total bacteria are presented in Figures 2.2, 2.3, and 2.4, respectively. In the phylogram for Accumulibacter, sequence Acc13 was excluded since it corresponded to γ proteobacteria. *Candidatus* Accumulibacter phosphatis, *Rhodocyclus tenuis* and *Dechloromonas agitata* were included as reference sequences (Fig. 2.2). For *Geobacteraceae*, the sequences of *Geobacter metallireducens, Pelobacter propionicus* and *Anaeromyxobacter sp.* were used as a reference (Fig. 2.3). In the case of bacterial 16S rRNA, sequences Total 27 and Total 29 appeared phylogenetically very far from the rest of the sequences, thus creating a large dispersion in the tree distances. Therefore, they were exluded in a subsequent analysis, which resulted in the phylogram presented in Fig. 2.4. The sequences of *Candidatus* Accumulibacter phosphatis and *Geobacter* were included in this phylogram, as well as the sequences of *Anaeromyxobacter*, a close match found for the *Geobacteraceae* library, as well as *Asticcacaulis excentricus, Ferribacterium limneticum and Terrimonas ferruginea,* which were found as close matches for the library of total bacteria. Furthermore, the sequences of *Thiobacillus denitrificans* and *Desulfovibrio,* two known denitrifier and SO_4^{2-} reducer, respectively, were also included.

3.1.1. Accumulibacter

Table 2.2. Results from RDP - Classifier for 16S rRNA genes library of Accumulibacter. The number of sequences corresponding to each hierarchy level appears in parenthesis, and the corresponding sequences below the classification. Confidence threshold: 80%.

Domain: Bacteria (30)
Phylum: Proteobacteria (30)
Class: β Proteobacteria (29)
Order: Rhodocyclales (28)
Family: Rhodocyclaceae (28)
Genus: Dechloromonas (2)
Acc2, Acc6
Genus: Propionivibrio (11)
Acc1, Acc3, Acc4, Acc8, Acc11, Acc16, Acc17, Acc18, Acc22, Acc26, Acc30
unclassified Rhodocyclaceae (15)
Acc5, Acc7, Acc9, Acc12, Acc14, Acc15, Acc19, Acc20, Acc21, Acc23, Acc24, Acc25, Acc27, Acc28, Acc29
unclassified β Proteobacteria (1)
Acc10
Class: γ Proteobacteria (1)
unclassified γ Proteobacteria (1)
Acc13

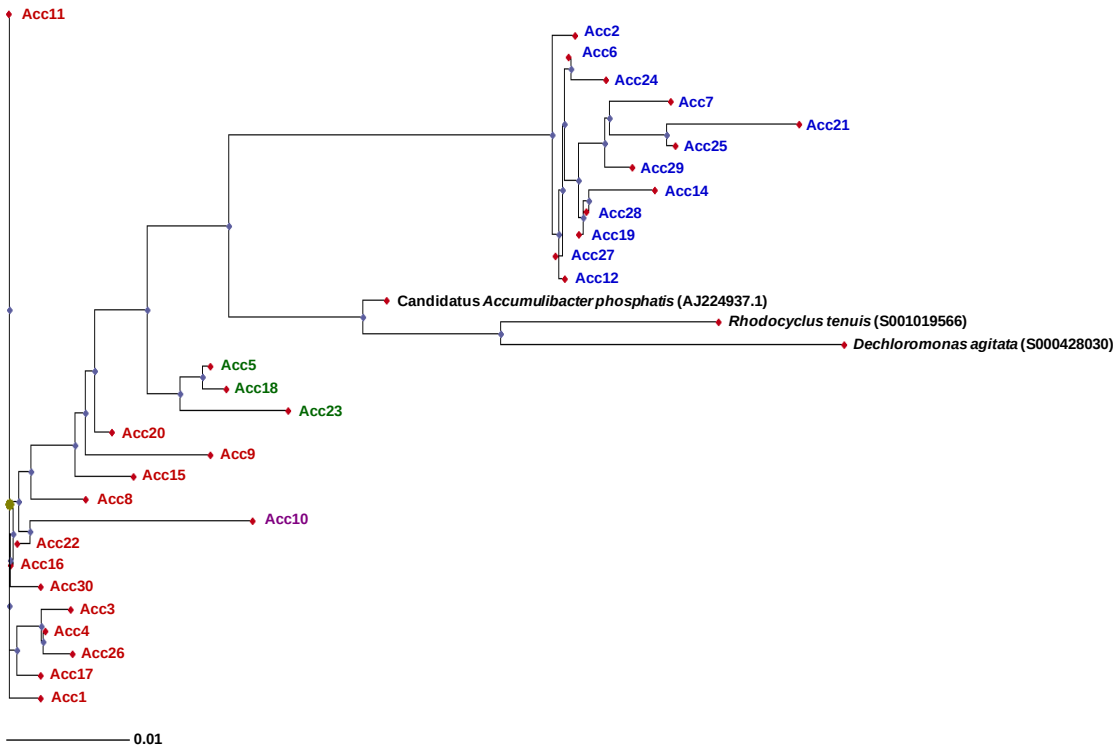

Figure 2.2. Phylogram for Accumulibacter. The tree shows the 5 OTUs found in this clone library, with each OTU of at least two sequences in a different color. Labels in black indicate those OTUs with a single sequence. Accession numbers of the reference sequences appear in parenthesis following the name.

3.1.2. Geobacteraceae

Table 2.3. Results corresponding to the top matches from BLAST for the nifD sequences of the *Geobacteraceae* library. The number of sequences corresponding to each hierarchy level appears in parenthesis, and the corresponding sequences below the classification. Maximum identity ≥ 74%.

Domain: Bacteria (28)
Phylum: Proteobacteria (27)
Class: δ proteobacteria (25)
Order: Desulfuromonales (10)
Family: *Geobacteraceae* (10)
Genus: *Geobacter* (6)
Species: *Geobacter bemidjiensis* (2)
Geo22, Geo30
Geobacter metallireducens (1)
Geo18
Uncultured *Geobacter* sp. clone PLYNIFD NifD (nifD) gene (3)
Geo11, Geo20, Geo27
Genus: *Pelobacter* (4)
Species: *Pelobacter propionicus* (4)
Geo10, Geo24, Geo28, Geo29
Order: Myxococcales (3)
Family: Cystobacteraceae (3)
Genus: *Anaeromyxobacter (3)*
Geo8, Geo21, Geo26
Uncultured δ proteobacterium clones NifD genes (12)
Geo1, Geo2, Geo5, Geo6, Geo7, Geo9, Geo12, Geo13, Geo14, Geo16, Geo17, Geo25
Unclassified Proteobacteria: *Magnetococcus* (2)
Geo15, Geo19
Phylum: Actinobacteria (1)
Class: Actinobacteria (1)
Order: Actinomycetales (1)
Family: Frankiaceae (1)
Genus: *Frankia* (1)
Geo23

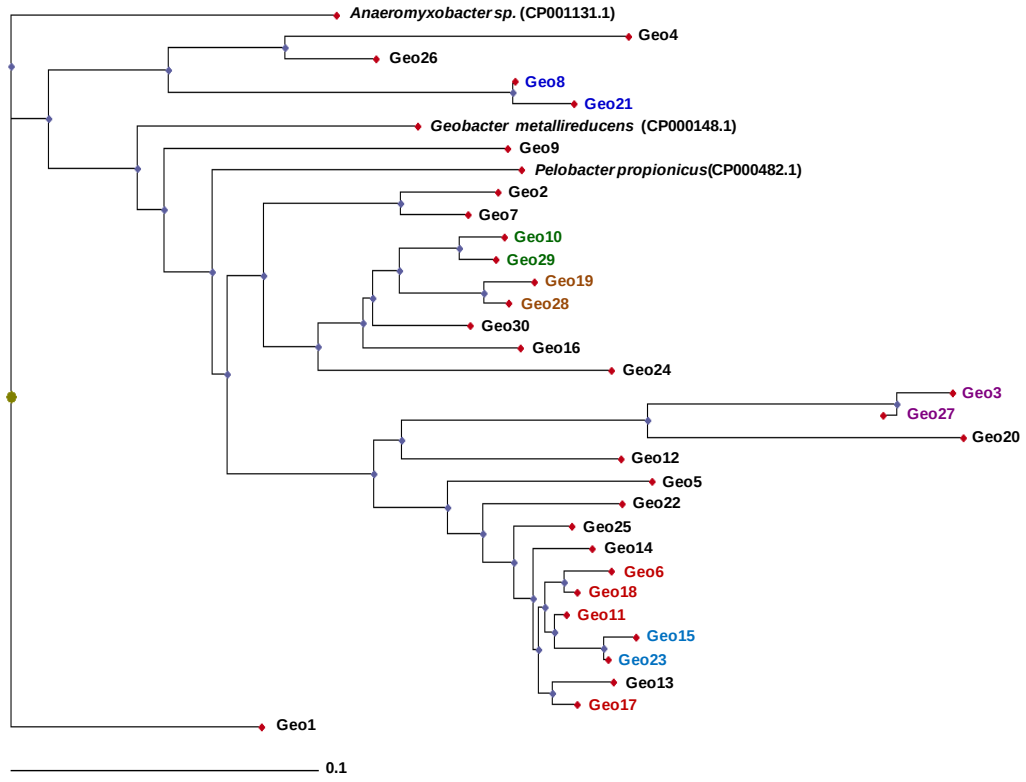

Figure 2.3. Phylogram for *Geobacteraceae*. The tree shows the 22 OTUs found in this clone library, with each OTU with at least two sequences in a different color. Labels in black indicate those OTUs with a single sequence. Accession numbers of the reference sequences appear in parenthesis following the name.

3.1.3. Total bacteria

Table 2.4. Results from RDP - Classifier for 16S rRNA genes library of total bacteria. The number of sequences corresponding to each hierarchy level appears in parenthesis, and the corresponding sequences below the classification. Confidence threshold: 80%.

Domain: Bacteria (29)
- **Phylum: Proteobacteria (11)**
  - **Class: α Proteobacteria (1)**
    - Order: Caulobacterales (1)
      - Family: Caulobacteraceae (1)
        - Genus: Asticcacaulis (1)
          - Total17
  - **Class: β Proteobacteria (5)**
    - Order: Rhodocyclales (3)
      - Family: Rhodocyclaceae (3)
        - Genus: *Ferribacterium* (1)
          - Total20
        - unclassified Rhodocyclaceae (2)
          - Total3, Total24
  - unclassified β Proteobacteria (2)
    - Total6, Total10
  - **Class: γ Proteobacteria (2)**
  - unclassified γ Proteobacteria (2)
    - Total13, Total25
- unclassified Proteobacteria (3)
  - Total1, Total8, Total23
- **Phylum: Bacteroidetes (3)**
  - **Class: Sphingobacteria (1)**
    - Order: Sphingobacteriales (1)
      - Family: Crenotrichaceae (1)
        - Genus: *Terrimonas* (1)
          - Total14
- unclassified Bacteroidetes (2)
  - Total19, Total30
- **Phylum: Firmicutes (1)**
  - **Class: Clostridia (1)**
    - Order: Clostridiales (1)
      - unclassified Clostridiales (1)
        - Total7

unclassified Bacteria (14)
Total2, Total4, Total5, Total9, Total11, Total12, Total15, Total16, Total18, Total21, Total22, Total27, Total28, Total29

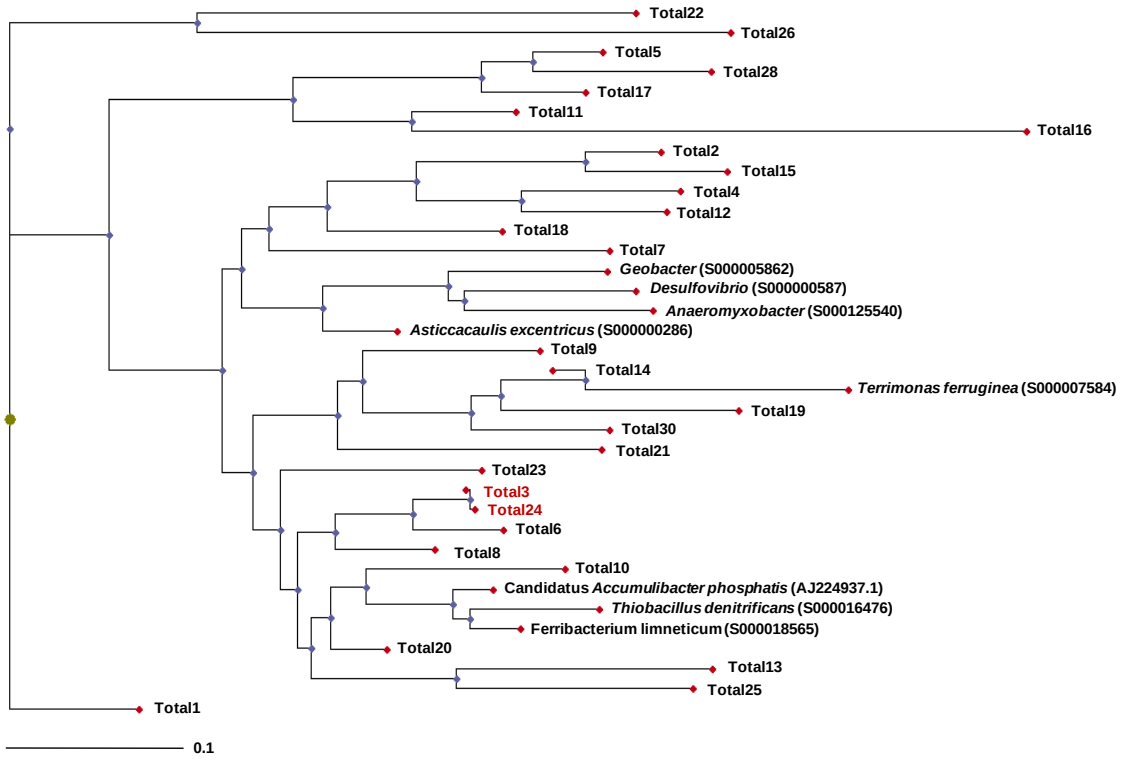

Figure 2.4. Phylogram for total bacteria. A total of 29 OTUs were found in this clone library, with two of the sequences being identical (in red). Accession numbers of the reference sequences appear in parenthesis following the name.

3.2. Quantitative PCR (qPCR)

The qPCR efficiency for total bacteria and *Geobacteraceae* was very good, although rather low for Accumulibacter (Table 2.5). The standard curve r^2 for the three groups, however, was very high, supporting the reliability of the data.

Table 2.5. qPCR efficiency and standard curve (r^2) for Accumulibacter, *Geobacteraceae* and total bacteria.

Group	qPCR Efficiency [a]	Standard Curve r^2
Accumulibacter	0.64	0.997
Geobacteraceae	1.04	0.994
Total bacteria	1.04	0.999

[a] Calculated as $10^{(-1/slope)} - 1$, based on the slope of the standard curve.

Average and standard deviations of qPCR for all the samples are in Appendix II. The qPCR analysis of the leachate samples discharged by the pipes indicates the presence of both Accumulibacter and *Geobacteraceae* groups, although in low numbers when compared to total bacteria (Table 2.6). The pipes were numbered according to their location from west to east. qPCR results corresponding to the field samples were processed and presented in the next section.

For both Accumulibacter and *Geobacteraceae* the biomass was very low in all the samples analyzed with the exception of a peak observed at the end of flooding cycle 3 in core 6 amended with glucose. The same peak was observed for total bacteria, whose biomass fluctuated randomly in the rest of the soil cores.

Table 2.6. Biomass (cells / mL leachate) of Accumulibacter, *Geobacteraceae* and total bacteria.

	Cells / mL leachate		
Leachate sample	**Accumulibacter**	***Geobacteraceae***	**Total bacteria**
Pipe 1	1.55E+05	8.39E+04	1.53E+09
Pipe 2	5.34E+05	n.d.[b]	8.07E+08
Pipe 3	1.32E+04	2.51E+03	7.04E+07
Pipe 4	4.21E+04	n.d.[b]	1.11E+08

[b]n.d.: Non-detectable.

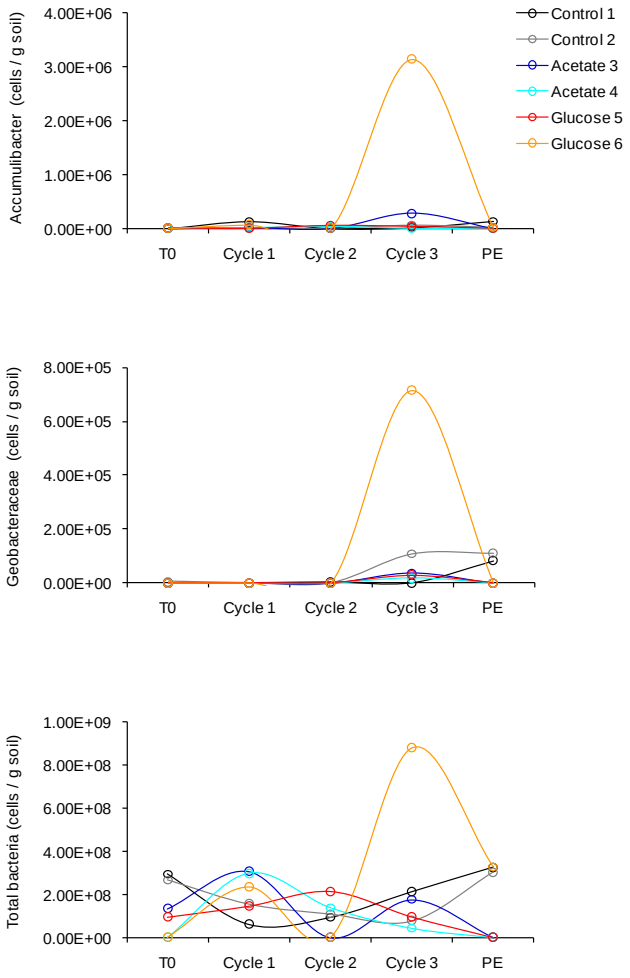

Figure 2.5. Biomass (cells / g soil) of Accumulibacter, *Geobacteraceae* and total bacteria in the soil cores.

3.3. Spatial distribution of bacterial groups

Accumulibacter is distributed in the entire field site, with some areas of high biomass located within the VFS cells, with a path running between the cells from north to south, following the terrain slope and ending in a large area with high biomass (Fig. 2.6).

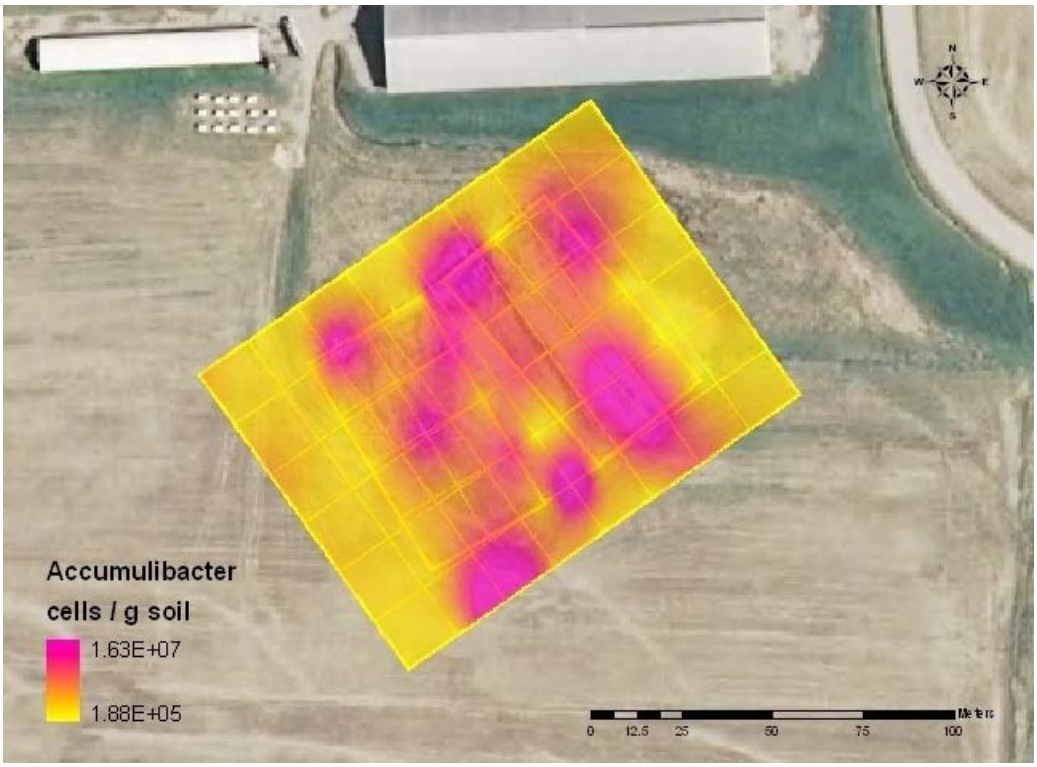

Figure 2.6. Spatial distribution of Accumulibacter in the field site.

In contrast to Accumulibacter, *Geobacteraceae* is clustered in two particular areas (Fig. 2.7). The smaller one, located in the perimeter, is probably an interpolation artifact. The larger area is located at the entrance of the right VFS cell, which according to field observations is often the wettest location in the field site. It is worth to notice that soils around pipe 1 diplay almost no *Geobactaceae* biomass, in spite of the high concentrations of this group found in the leachate discharging in this particular location (Table 2.6).

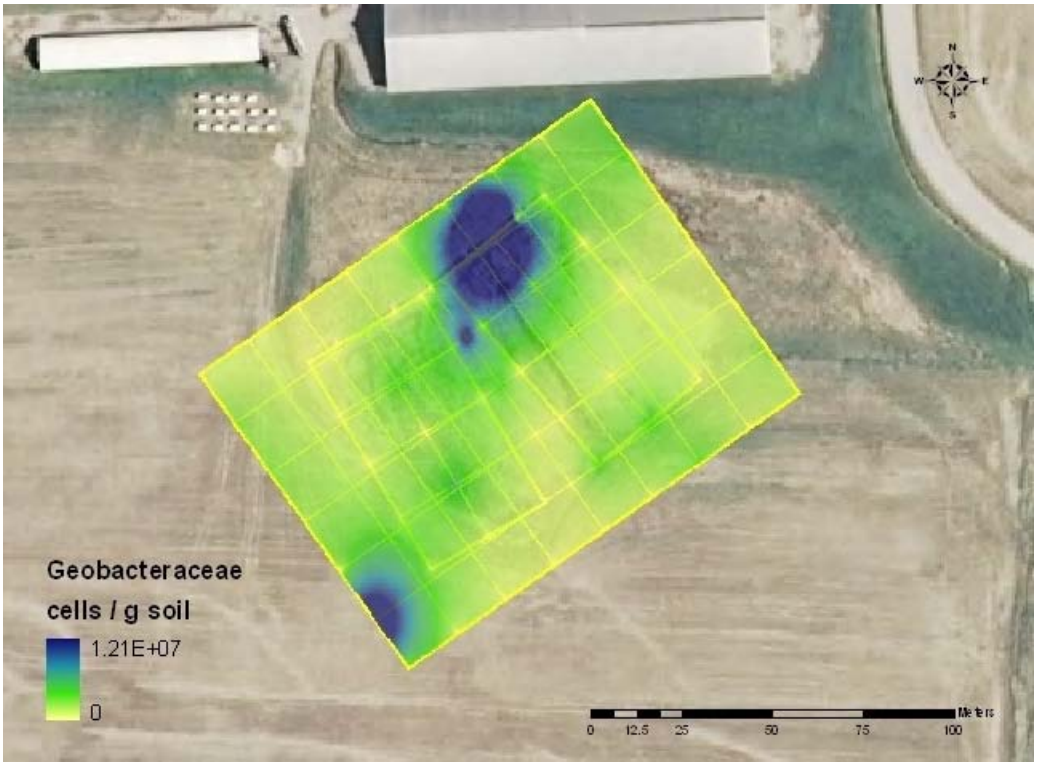

Figure 2.7. Spatial distribution of *Geobacteraceae* in the field site.

High numbers of total bacteria were present in the entire filed site (Fig. 2.8). A notorious hot-spot is visible at the entrance of the left VFS cell, as well as a path exhibiting a high bacterial biomass similarly to Accumulibacter.

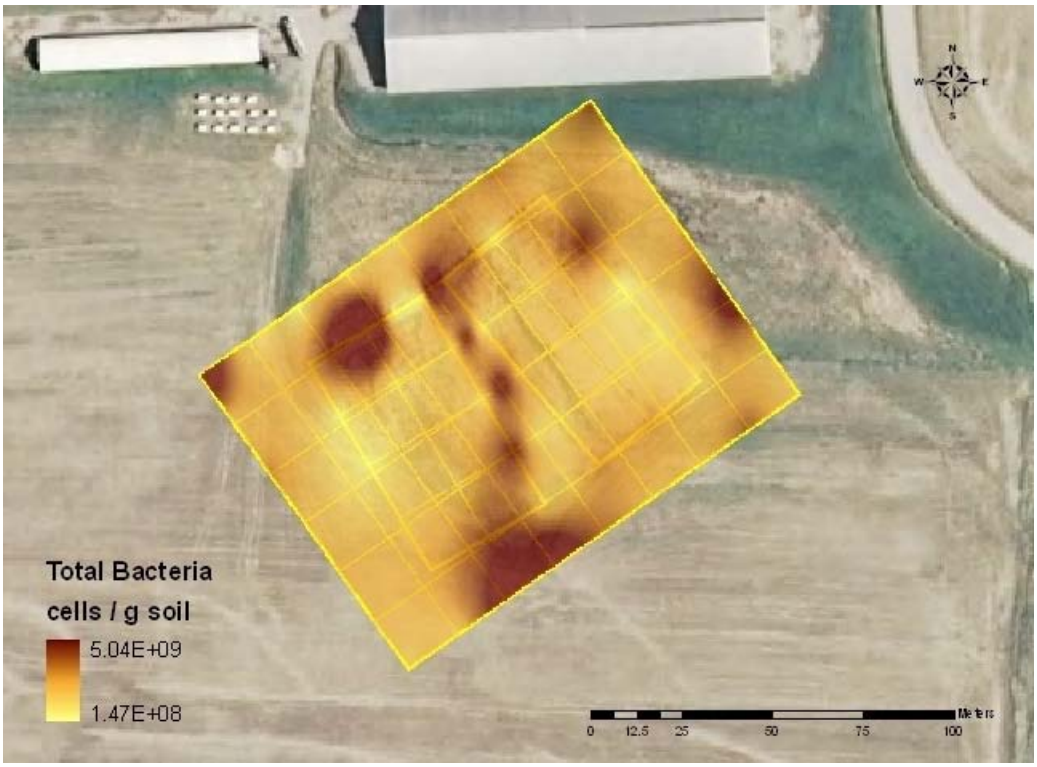

Figure 2.8. Spatial distribution of total bacteria in the field site.

4. Discussion

4.1. Phylogenetic analysis

The initial screening of the samples revealed the presence of Accumulibacter and *Geobacteraceae* in the soils of the study site. These findings are very significant although not entirely surprising. Accumulibacter has been recently found in freshwater and associated sediments, and also, although rarely, in soils (Kunin et al., 2008; Peterson et al., 2008). *Geobacteraceae*, on the other hand, has long been found as a ubiquitous group in many environments, mostly sedimentary.

It has been proposed that the presence of Accumulibacter in environmental samples is the result of the dispersion of these microorganisms from open activated sludge basins enriched via aerosols (Kunin et al., 2008), being then further spread though aquatic reservoirs (Peterson et al., 2008). A remarkable aspect of the present study is that the field site is located in Freeville, Tompkins County, NY, inserted in an agricultural landscape, far away from any WWTP, with no known history of soil amendments with activated sludge. This means that Accumulibacter probably naturally occurs in this particular location and is, very likely, distributed in the environment.

The clone library of Accumulibacter revealed that 29 sequences, with only one exception, corresponded to β Proteobacteria within the family *Rhodocyclaceae*, including the genus *Dechloromonas and Propionivibrio.* The remaining sequence was classified as γ proteobacteria. These primers were

developed for targeting Accumulibacter in EPBR systems highly enriched with this microorganism (He et al., 2007). Their specificity was tested *in silico* at the RDP Database Probe-Match (http://rdp.cme.msu.edu/probematch/search.jsp) and they indeed targeted exclusively β Proteobacteria (data not shown). It is possible that their use in soil derived genomic DNA might have resulted in a change in their specificity, given the highest microbial diversity expected in such a complex matrix.

In the clone library of *Geobacteraceae* only 10 of the sequences were classified within this family. The primers used were originally developed for targeting *Geobacteraceae* in subsurface environments where this group is dominant, e.g. Holmes et al. (2004b, 2007). Their specificity was tested *in silico* at Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and targeted exclusively *Geobacter* species (data not shown). Similarly to the case of Accumulibacter, the use of these primers in soils probably also resulted in a change in their specificity. Interestingly, three of the remaining sequences correspond to *Anaeromyxobacter*, another known Fe reducer, which, similarly to *Geobacteraceae*, has also shown the ability to oxidize acetate (Hori et al., 2007).

The clone library for total bacteria was composed by 16 sequences of unknown bacteria, plus sequences of α, β and γ proteobacteria, bacteriodetes and firmicutes. β protobacteria were represented by *Rhodocyclaceae*, most of them unclassified, in addition to one sequence corresponding to *Ferribacterium*. *Ferribacterium limneticum* is a motile, obligately anaerobic rod isolated from mine wastes and enriched with acetate and Fe(III)-oxyhydroxide,

being the first dissimilatory Fe(III) reducer in the β subclass of proteobacteria (Cummings et al., 1998). Thus, Fe reduction seems to be an important microbially mediated process in the field site, and likely not exclusively attributable to *Geobacteraceae.*

4.2. Quantitative PCR (qPCR)

qPCR efficiency was relatively low for Accumulibacter in contrast with the other two groups. Amplification efficiency for qPCR is generally calculated from the slope of the log-linear region, assuming that log-linearity reflects constant amplification efficiency. Rutledge and Stewart (2008), however, demonstrated that SYBR Green derived amplification profiles lack the exponential character often ascribed to qPCR. Consequently, methods that rely on such assumption generate underestimations in the extent of the bias and variability of the resulting amplification efficiency.

Both Accumulibacter and *Geobacteraceae* were found in the samples taken from the six soil cores used in the experiment described in Chapter 1 (Fig. 2.5), constituting important evidence of their potential impact on the results. Nevertheless, the analysis with ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR) revealed the absence of polyP in the soil cores amended with acetate, which went through three 10-day flooding cycles and were subsequently aerobically enriched with P. These experiments suggested that PAOs, specifically Accumulibacter, were indeed present in the soil cores but not actively uptaking P and storing intracellular polyP. Nevertheless, the analysis of soils from the cores amended with glucose with ^{31}P-NMR, which

was not performed, could have provided an important piece of additional evidence. The presence of *Geobacteraceae,* in contrast, agreed with observed Fe reduction during the flooding in the cores containing glucose, as described in Chapter 1. Nevertheless, for these two groups, there are no apparent trends in terms of biomass over the flooding cycles (Fig. 2.5). Furthermore, the biomass of total bacteria is not higher in the cores containing a C source, as might have been expected. The peak observed for the three primer sets used in Core 6 at the end of Cycle 3 is probably an anomalous point, since qPCR triplicate values are similar (data not shown). It is likely the result of an underestimation of the DNA amount extracted from that particular soil sample. In general, it is possible that the samples taken from the cores were not really representative, since, in order to minimize any possible disturbance, very small samples were taken only from the surface soil. In addition, they were stored at -20°C for several months before their DNA was extracted and analyzed, thus degradation during storage cannot be ruled out.

Accumulibacter and *Geobacteraceae* were also found in the leachate samples (Table 2.6). While Accumulibacter was found in all four samples, *Geobacteraceae* was only found in two of them. Silage leachate contains high concentrations of acetic acid as the result of the fermentation process intentionally promoted by covering bunker silos to preserve the starting material (Denoncourt et al., 2007). The anaerobic conditions promoted plus the presence of acetate might favor the proliferation of both groups in the leachate. It is unclear, however, if their ultimate origin is the soil or the silage itself. Total bacteria were found in high numbers in all the leachate samples.

4.3. Spatial distribution of bacterial groups

A significant aspect of the present study is that is that, although Accumulibacter and *Geobacteraceae* have been previously found in natural environments, it constitutes the first report of spatial distribution patterns of both groups in soils, with the potential of relating such patterns to hydrological and pedological features in the landscape. From these results emerges a new approach to study potential microbial controls on soil P dynamics.

Accumulibacter was present in all the soil samples taken, with the highest biomass within the VFS cells (Fig. 2.6), whose soils have, in general, a higher moisture content than the surrounding area; the bottom of the VFS is also, not surprisingly, noticeably wet and coincides with PAO abundance. Silage leachate is constantly discharged in the VFS, thus defining flow paths that are shaped by vegetation and the terrain slope. The discharge, however, does not occur at constant flow, thus resulting in a hydrological pulse that will cause soil saturation and draining of particular spots within short temporal scales, i.e. hours to days. Such conditions might favor the development of Accumulibacter populations, giving the fluctuating redox potentials similar to those required in EBPR systems.

The distribution of *Geobacteraceae*, in contrast, was clustered in two locations and absent in many of the soil samples analyzed (Fig. 2.7). A significant high biomass spot is visible at the entrance of the VFS cell 2, which according to field observations is one of the consistently wettest locations on the study site. This particular area is directly receiving most of the silage leachate, which

contains a high biomass of *Geobacteraceae* (Table 2.6), hence the soils here resemble sediments since they are not only almost permanently flooded but also largely composed of finer soil particles compared to the rest of the site, thus, it is arguably similar to submerged environments where *Geobacteraceae* has been generally found. That might explain why this group is barely present in the rest of the field where soils are dryer and also have larger pores.

Total bacteria were present in high numbers over the entire study site. A significant feature of the distribution pattern is a distinctive transect with high bacterial biomass between the cells of the filter strip, oriented from north to south (Fig. 2.8). According to field observations, runoff follows the terrain slope though that particular flow path, suggesting that soil bacteria are hydrologically transported in that direction, probably bound to eroded soil particles, and perhaps thrive in this regularly wetted corridor. Soil bacterial biomass, as well as diversity, is strongly and positively correlated with soil moisture (Banu et al., 2004). A somewhat similar pattern, although not obvious in Fig. 2.6, was also observed for Accumulibacter.

There are several aspects of Accumulibacter physiology that may result in an advantage for its survival and persistence in natural environments, including soils. Recently, the retrieval of near-complete genomes of Accumulibacter from lab-scale EBPR enrichment culture using shotgun sequencing allowed researchers to perform a comprehensive metabolic reconstruction of this organism (Garcia et al., 2006). These authors found multiple genes encoding functions more likely to be used in environments different than activated sludge systems, including complete pathways for nitrogen and carbon fixation

and high affinity phosphate transporters, indicating the adaptation of this organism to nutrient limited conditions. In addition, the presence of flagellar genes might confer Accumulibacter the capability of moving within aquatic environments, thus facilitating its dispersal in natural ecosystems.

Further advantages for Accumulibacter survival and persistence in soils might emerge from features of polyP metabolism. The enzyme ppk1, responsible for intracellular polyP formation, has proven to be crucial for biofilm development (Rashid et al., 2000). Biofilms are essential for the survival of many bacterial species (Stoodley et al., 2002), particularly for those inhabiting soils. The hydrophilic surface of biofilms provides protection against predation by environmental protozoa (Hall-Stoodley et al., 2004), and the produced exopolymers attenuate UV light penetration, thus reducing the risk of desiccation (Elasri and Miller, 1999). In addition, a fraction of the biofilm population acquires extreme phenotypic resistance to numerous stressors (Brown and Smith, 2003; Shiba et al., 1997). PolyP is indeed involved in the transcriptional responses of numerous genes that shift growth and metabolism towards cell protection and dormancy as a general stress response (Hengge-Aronis, 2002). Among the main stressors for bacteria, osmotic pressure is one of the strongest, since desiccation is potentially lethal. The lack of ppk1 in *Pseudomonas aeruginosa* reduces survival during desiccation, both for biofilms and planktonic cells (Fraley et al., 2007). A further line of evidence linking polyP with the ability of bacterial cells to withstand osmotic pressure is constituted by recent functions discovered for acidocalcisomes in prokayiotes (see review by DoCampo et al., 2005). Acidocalcisomes are electron-dense acidic organelles rich in calcium and polyP, which are the only ones conserved

during evolution from prokaryotes to eukaryotes. These organelles, previously known as volutin or metachromatic granules and polyP vacuoles, have been linked with several functions related to polyP metabolism and osmoregulation, among others. Rapid hydrolysis or synthesis of acidocalcisomal polyP occurs when cells of the prokaryotic parasite *Trypanosoma cruzi* are exposed to hypoosmotic or hyperosmotic stress conditions, respectively (Ruiz at al., 2001), indicating a link between acidocalcisomes and osmotic homeostasis. This link has been additionally supported by the response of the cells of another prokaryotic parasite, *Leishmania major,* which show changes in the sodium and chlorine content of the acidocalcisomes after hypoosmotic stress (LeFurgey et al. 2001).

Perhaps one of the most remarkable roles of polyP is in adaptive evolution, which might explain the global distribution of Accumulibacter reported by Kunin et al. (2008). Error-prone DNA replication induced by environmental stress can lead to increased genetic variation, which might lead to the existence of some strains that are more suited to survival in particular conditions (Brown and Kornberg, 2008). ppk1 regulates error-prone DNA replication by DNA polymerase IV, which has shown to lead to adaptive mutation in *Escherichia coli* (Stumpf and Foster, 2005). This might certainly contribute to the adaptation of *Accumulibacer* to different environments that impose diverse stressors on its local populations.

Similarly, there are several features in *Geobacteraceae* metabolism that make this family of bacteria able to successfully persist in and dominate subsurface environments. The capability of fixing nitrogen seems to be widespread in this

group, since all of thirty *Geobacteraceae* species studied have the gene that encodes the α subunit of the dinitrogenase protein, nifD (Holmes et al., 2004a), some of which have also demonstrated this capability experimentally (Bazylinski et al., 2000; Coppi et al., 2001). Thus, this group may adapt and proliferate in nutrient limited environments, unlike other well studied species of Fe reducers lacking the nifD gene, e.g. *Shewanella oneidensis* (Heidelberg et al., 2002) and *Desulfovibrio vulgari* (Heidelberg et al., 2004). Another uniqueness of this group is that *Geobacter* species must contact Fe oxides in order to reduce them, while other Fe reducers, e.g. *Shewanella alga* (Nevin and Lovley, 2002), are able to produce extracellular, soluble Fe chelators and electron shuttles, thus eliminating the need of physical contact with Fe oxides for reduction (Nevin and Lovley, 2000, 2002; Newman and Kolter, 2000). While this strategy is probably effective under certain circumstances, it is energetically expensive, with a cost that would quickly become a net energy loss for the organism if is used on regular basis. Thus, *Geobacter* species might be energetically favored proven the direct contact with the substrate.

A common component of the metabolism of *Accumulibacer* and *Geobacteraceae* is that both groups use acetate preferentially over other electron donors. This might constitute a competitive advantage given that acetate has been identified as the main intermediate in anaerobic mineralization of organic C in many aquatic ecosystems (Lovley and Klug, 1982; Rothfuss and Conrad, 1993), playing also an important role in upland environments, such as prairie and forest soils, where anaerobic conditions are restricted to microsites in soil aggregates (Küsel and Drake, 1994; Wagner et al., 1996).

4.4. Ecological significance of Accumulibacter and polyP metabolism

The presence of Accumulibacter and *Geobacteraceae* in the study site constitutes an important line of evidence for microbial controls on soil P dynamics. In the case of Accumulibacter, as mentioned above, the lack of evidence for polyP accumulation in the soil cores under conditions known to promote EBPR suggest that the other factors might act as controls for activating polyP metabolism, such as flanking bacterial populations and quorum sensing. Flanking microbial populations found in EBPR reactors dominated by Accumulibacter have been proposed to provide nitrite for *Accumilibacter* anaerobic respiration (Garcia et al., 2006). Synthopic relations, i.e. when one species lives on the products of another, seem therefore to be very important for the survival and predominance of Accumulibacter, an organism that, to date, has not been isolated but only enriched at high numbers (e.g. Lu et al., 2006). Quorum sensing, on the other hand, is an important environmental monitoring system in which cell density and the associated secretion of specific signal compounds, commonly lactones, coordinates the response of a bacterial population (Parsek and Greenberg, 2005; Schuster and Greenberg, 2007; Williams, 2007). It is possible that this mechanism may somehow coordinate the ability of Accumulibacter to activate polyP metabolism and accumulate intracellular polyP once a threshold cell density has been reached. Further research is required, however, in order to test these speculative theories.

One thing is for sure: polyP metabolism does indeed occur in nature. Evidence of biogenic polyP in sediments using ^{31}P Nuclear Magnetic Resonance

Spectroscopy (^{31}P-NMR) supports the ecological role of these microorganisms in P dynamics (Hupfer et al., 1995, 2004; Carman et al., 2000; Khoshmanesh et al., 2002; Reitzel et al., 2006, 2007). No molecular PCR based techniques were used in those studies, however, thus the identity of the microorganisms responsible of such process remains unknown. A further line of evidence for the ecological role of polyP in natural ecosystems is the formation of major deposits of phosphorites in highly productive upwelling areas along the coast of Namibia, which has been recently related with the presence of a high biomass of the giant marine sulfur bacterium *Thiomargarita namibiensis* in surface sediments by Schulz and Schulz (2005). These sulfur bacteria species contain large amounts of intracellular polyP, and based on incubation experiments these researchers have hypothesized that the mechanism of P uptake and release in this species is similar to that found in EBPR systems. This bacterium periodically contacts oxic bottom water in order to take up and store nitrate, which allow it to survive long intervals of anoxia (Shultz et al., 1999) while releasing P. As observed experimentally, this release can concentrate pore water phosphate high enough to drive spontaneous precipitation of phosphorus minerals.

PolyP in the biomass of sediment microorganisms as a temporal sink of P has been controversially discussed. One of the main reasons is that the relevance of microbial biomass is considered by some researchers arguable for being responsible of a significant P release, e.g. Golterman (2004). It is necessary to acknowledge, however, that microbial and chemical processes regulating P cycling are not mutually exclusive but in fact likely coupled. The results of Schulz and Schulz (2005) support that. Thus, it is likely that the role of

Accumulibacter and other unknown PAOs finds a niche in the formation of P minerals, rather than constituting a P sink in the microbial biomass itself. Such ecological functions remain, now, only speculations, until further studies are conducted.

5. Conclusions

The conclusions of this study are the following:

i) Accumulibacter and *Geobacteraceae* were present in the soils of the study site, constituting a line of evidence of their potential role in soil P dynamics.

ii) The PCR primers used for Accumulibacter showed a higher specificity in comparison to the ones corresponding to *Geobacteraceae*, as shown by the sequences in the clone libraries. The constructed libraries, including the one for total bacteria, contained several sequences of Fe reducers other than *Geobacteraceae*, indicating that Fe reduction might be a significant process in the study site and not exclusively restricted to the role of this group.

iii) The spatial distribution of Accumulibacter showed that this microorganism was dispersed over the entire field site, with apparently higher biomass within the VFS cells likely promoted by the higher moisture compared to the rest of the field. In contrast, *Geobacteraceae* was clustered in particular areas that resemble the sedimentary environments where this group has often been found. The biomass of total bacteria was high in the entire site, showing some evidence of bacterial transport with runoff and affinity for areas where shallow soil water accumulates.

iv) Both groups, Accumulibacter and *Geobacteraceae,* were present in the soil cores from a previous experiment, with no apparent or consistent trends in terms of biomass over the duration of the experiment. They were also present in the silage leachate samples, making unclear if their ultimate origin is the silage or the soil itself.

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CHAPTER 3

Biogeochemical and Microbial Aspects of Soil Phosphorus Dynamics: A Conceptual Framework

Abstract

Soil P transformations are strongly influenced by hydrology, since water not only provides a medium for chemicals dissolution and transport, but also enhances biogeochemical activity. Thus, many of the strategies for minimizing non point source (NPS) P pollution, which are the leading cause of freshwater eutrophication, rely on intercepting runoff waters with the purpose of retaining and transforming the different P forms, and are thus denominated buffer zones. Vegetated Filter Strips (VFSs) are one of the most commonly used, although their effectiveness has long been the subject of controversy, since such systems accumulates P until they reach their maximum retention capacity. Thus, degradation plus the strategic locations of VFSs make these systems highly susceptible to flooding, becoming then prone to release P and become P sources. They often receive a number of other nutrients and pollutants, thus exhibiting a vast array of simultaneous biogeochemical transformations potentially significant in P cycling. For this reason, the field site chosen for conducting the research on soil P dynamics described in Chapters 1 and 2 was a VFSs located on a large Concentrated Animal Feeding Operation (CAFO) dairy farm designed to treat silage leachate. Further aspects and implications of the findings of this research are discussed in the present paper.

1. Introduction

The persistence of freshwater eutrophication as a serious water quality problem has resulted in countless studies conducted over the years in order to gain a better understanding of the processes regulating P transport and dynamics in terrestrial and aquatic ecosystems. As a consequence of the inherent complexity of P cycling, its different aspects have often been approached independently, generating thus partial views of a phenomenon that can certainly not be divided into pieces.

There is a consensus about the importance of hydrology in P fluxes. Water not only provides a medium for chemicals dissolution and transport, but also enhances biogeochemical activity. Areas with disproportionately high reaction rates relative to the surrounding matrix have been defined by McClain et al. (2003) as biogeochemical hot-spots. They commonly occur at the boundary or ecotone between two features in a landscape. In aquatic ecosystems, dynamic P transformations often take place in surface sediments. Similarly, enhanced P release has been observed in areas in the landscape prone to saturate and hence to flooding and to produce runoff. Such areas exhibit an enhanced hydrologic sensitivity relative to non runoff generating areas, since runoff provides a rapid transport mechanism for nutrients and potential pollutants between the soils and surface water bodies (Walter et al., 2000). These areas are appropriately called Hydrologically Sensitive Areas (HSAs), and are dominated by Variable Source Area (VSA) hydrology, a watershed process whereby saturated areas are the primary sources of runoff. This name was originally attributed to Hewlett and Hibbert (1967), but the concept was further

developed by Dunne (1970), Dunne and Black (1970), Hewlett and Nutter (1970) and Dunne et al. (1975).

Many of the strategies for minimizing non point source (NPS) P pollution, which have been identified as the leading cause of freshwater eutrophication, rely on intercepting runoff waters with the purpose of retaining and transforming the different P forms (Dorioz et al., 2006). These landscape features are denominated buffer zones, and Vegetated Filter Strips (VFSs) are one of the most commonly used. The effectiveness of VFSs, however, has long been the subject of controversy, since there is no biogeochemical transformation capable of reducing the amount of P stored in soils. Thus, it accumulates in such systems until they reach their maximum retention capacity and can no longer store additional P, becoming then prone to release it. The implications of this observation are noteworthy, since given their strategic location in the landscape, these areas are highly susceptible to flooding, and thus may paradoxically become P sources. One remarkable aspect of VFSs in terms of P transformations is that they also capture a number of other nutrients and pollutants, thus exhibiting a vast array of simultaneous biogeochemical transformations potentially significant in P cycling. For this reason, the field site chosen for conducting the research on soil P dynamics described in Chapters 1 and 2 was a VFSs located on a large Concentrated Animal Feeding Operation (CAFO) dairy farm designed to treat silage leachate. Further aspects and implications of the findings of this research are discussed in the present paper, whose main purpose is to provide a conceptual framework of soil P dynamics.

2. Theoretical Background

2.1. Physical-chemical processes involved in P cycling

2.1.1. Sorption

Sorption is defined broadly as the transfer of ions from the solution phase to the solid phase. Anions in solution are retained in soils primarily by selective bonding at variable charge minerals and layer silicate particle edges. This process, defined as chemisorption (McBride, 1994), implies a strong adsorbate-adsorbant interaction, in which a covalent or short range electrostatic bond forms between the molecule and the surface.

The presence of amorphous and hydrous oxides of iron (Fe), manganese (Mn) and aluminum (Al) in soils has long been considered as one the most important factors affecting P retention. The classic Einsele-Mortimer model (Einsele, 1936, 1938; Mortimer, 1941, 1942) emphasizes the importance of the redox-dependent reduction of Fe oxides in P cycling. Fe reduction is indeed considered the dominant source of P release in flooded soils (Willett, 1989; Shahandeh et al., 2003). While the effects of Mn and Al oxides on P retention are somehow similar, the importance of Mn in P release is limited to initial stages of soil reduction and to the presence of large amount of reactive Mn oxides (Shanahdeh et al., 2003), i.e. in soils with Fe : Mn content ratios less than five. Al reduction, on the other hand, is not redox sensitive, and thus Al oxides play a role only in terms of P retention by constituting a long a term P sink (Kopacek et al., 2005).

2.1.2. Precipitation

Soil P sorption displays a complex behavior since the sorption capacity seems to increase over time. This time-dependent phenomenon may be the consequence of slow precipitation reactions superimposed on the more rapid chemisorption (McBride, 1994). Indeed, evidence suggests that phosphate chemisorption and surface precipitation can occur simultaneously with Al (e.g. Khare et al., 2005) and Fe minerals (e.g. Ler and Stanforth, 2003). Thus, surface precipitation may increase the apparent sorption capacity of a mineral (Van Riemsdijk and Lyklema, 1979; Laiti et al., 1996). In addition, under such circumstances, some of the P is buried under the particle surface and thus isolated from the exchange solution, resulting in an inhibition of phosphate desorption (Li and Stanforth, 2000).

McBride (1994) describes two P precipitation models in soils according to pH. In acid mineral soils, phosphate solubility is limited to solubility of variscite ($AlPO_4 \cdot 2H_2O$). Thus, phosphate solubility is expected to decrease with acidification. However, in soils containing large amounts of humus or high-charge clays, e.g. smectite and vermiculite, this model does not apply and thus phosphate solubility can indeed increase as pH decreases, effect attributed to the rapid adsorption of Al^{3+} released by mineral dissolution, by organic and clay colloids. In nonacid soils, phosphate solubility is controlled by the precipitation of different forms of Ca phosphate, which become less soluble than Al phosphate as pH increases. This pH dependence, however, is limited in alkaline soils with high contents of exchangeable sodium (Na^{2+}) and elevated concentrations of bicarbonates and carbonates. In such conditions,

high Na^{2+} inhibits Ca phosphate precipitation and alkaline anions displace phosphate from chemisorptions sites on variable charge minerals.

2.2. Microbial processes involved in P cycling

2.2.1. Decay of soil microbial biomass

a) Osmotic shock

The flooding of soils after long term drying has shown to enhance P fluxes, which are dominated by organic forms (Turner and Haygarth, 2001). The physical stress induced by soil drying can disrupt organic matter coatings on clay and mineral surfaces (Bartlett and James, 1980), thus partially contributing to such fluxes (Turner et al., 2002). Evidence indicates, however, that the primary source of organic P under those circumstances is the decay of soil microbial biomass (Turner and Haygarth, 2001). Rapid rehydration can kill between 17% and 58% of soil microbes by inducing cell rupture due to osmotic shock (Salema et al., 1982; Kieft et al., 1987). This effect immediately follows rewetting, with a time span of around 48 h (Turner and Haygarth, 2001).

b) Soil Microbial Community Shift

When intermittent flooding occurs, soils are often able to retain certain degree of moisture. Thus, soil microbes, rather than being exposed to an osmotic shock, are in fact selected based on their ability to withstand the initial osmotic

stress along with the anaerobic environment gradually promoted by oxygen depletion. This might generate a P flux dominated, as in the previous case, by organic forms derived from the decay of strictly aerobic microbes, as the result of a microbial community shift. Many microorganisms become dormant until conditions become more favorable (Schimel et al., 2007), explaining why some groups can proliferate in increasingly reducing conditions following flooding according to their life history strategies. The results reported in Chapter 1 support this theory, since a sustained P_o released was actually observed after flooding for as long as 10 days.

2.2.2. Polyphosphate (polyP) metabolism

a) Metabolic aspects of the EBPR Process

The Enhanced Biological Phosphorus Removal (EBPR) process has been extensively studied over five decades in laboratory scale sequencing batch reactors (SBRs), pilot-scale systems and full-scale wastewater treatment plants (WWTPs) (see review by McMahon et al., 2007). EBPR is performed by microorganisms properly called Polyphosphate Accumulating Organisms (PAOs), which are capable of phosphate uptake and intracellular storage of polyP under aerobic conditions. When the environment turns anoxic, the hydrolysis of stored polyP granules results in phosphate release with the concomitant uptake of organic acids, primarily acetate and propionate (Tchobanoglous et al., 2003). Most of the aspects of the engineering development and design of EBPR have been summarized by VanLoosdrecht et al. (1997), some of which are presented in this section.

EBPR was first noticed in a WWTP in India. Srinath et al. (1959) observed that the sludge exhibited excessive phosphate uptake under aeration. A few years later, the first structured investigation of this process was conducted by Levin and Shapiro (1965) in several treatment plants. They observed that the phosphate was taken up under aerobic conditions and stored in volutin granules, which was hypothesized to occur via formation of adenosine triphosphate (ATP) during oxidative phosphorylation. They also observed P release when the sludge was not aerated. In this and other studies from the period glucose was thought to be the main substrate, since fermentation products were not yet recognized.

Milbury et al. (1971) stated that the design of a reactor for EBPR should include a non aerated phase as a first step. The existence of an anaerobic phase was since then recognized as a prerequisite for an efficient P removal in the engineering approach that was developed mostly by Barnard (1974, 1975) and Nicholls (1975). Advances in the microbiological aspects of EBPR provided further insight to the fundamentals of the process. Fuhs and Chen (1975) proposed that the anaerobic phase was required to produce volatile fatty acids (VFAs) such as acetate that serve as substrates for the P removing organisms. Based on the results of a series of isolation tests, they postulated that bacteria of the genus *Acinetobacter* were responsible for EBPR. These organisms could grow aerobically using these substrates and uptaking P in excess, but they showed only a modest anaerobic P release compared to activated sludge.

Around that time, the attention turned to the aerobic P uptake. It was hypothesized that this overplus or luxury P uptake was performed by aerobic bacteria as a stress response to the anaerobic environment and the dynamic feeding in the activated sludge system. These observations led to a process design proposed by Nicholls and Osborn (1979) that was functionally correct although mechanistically wrong. The link between phosphate release and uptake was finally found when Rensink (1981) reported that substrate might be sequestered as polyhydroxybutyrate (PHB) by strictly aerobic organisms under anaerobic conditions at the expense of energy stored as polyphosphate. It then became clear that the main function of the anaerobic phase was not to just supply VFAs or provide a stress factor, but to give PAOs a competitive advantage for substrate uptake over other heterotrophic bacteria. This hypothesis became the basis for the biochemical framework further developed by subsequent researchers, which has to date completely relied on empirical observations in WWTP and lab scale bioreactors, since bacteria exhibiting all of the metabolic capabilities observed in EBPR have not yet been cultivated in isolation.

The consensus of the metabolic models for EBPR, as described by Garcia et al (2006), is that P_i is taken up from wastewater by PAOs and converted into polyP during the aerobic period. During the anaerobic period, these PAOs break the phosphodiester bonds of the stored polyP to provide an energy source for taking up VFAs, mostly acetate and propionate, and storing them as polyhydroxyalkanoates (PHA) such as PHB. The efficient anaerobic sequestration of VFAs would give PAOs a selective advantage over other members of the microbial community for subsequent growth and replication

during the aerobic period, allowing them to be the dominant group in EBPR systems

In recent years, the development and application of culture independent techniques has made possible important advances in the study of the microbiology of EBPR. It has been demonstrated that the genus *Acinetobacter*, although present in most EBPR systems, is not actually responsible for the process (Bond et al. 1995; Wagner et al., 1994; Mino et al. 1998). Furthermore, based on 16S rRNA analysis, the first confirmed PAO, *Candidatus* Accumulibacter phosphatis (Hesselmann, 1989), was finally identified. Fluorescence in situ hybridization (FISH) confirmed the dominance and role of this β protobacterium of the family *Rodocyclaceae*, in EBPR systems, including lab-scale reactors, pilot-scale systems, and full-scale wastewater treatment plants (WWTP) (see review by McMahon et al., 2007). Although it has not yet been grown in axenic culture, it has been enriched up to 90% of total cells in lab-scale bioreactors (Lu et al., 2006).

b) Evidence of PolyP metabolism in natural ecosystems

Redox fluctuations in surface sediments often result in P exchange between sediments and the anoxic water column. Such phenomenon, observed over the years, has been linked to the role of facultative bacteria capable of luxury aerobic P uptake and anaerobic P release, i.e. PAOs (Fleischer 1983, 1986; Gächter et al., 1988; Davelaar, 1993; Gächter and Meyer, 1993; Goedkoop and Pettersson, 2000; Khoshmanesh et al., 2002; Hupfer et al., 1995, 2004, 2007; Maassen et al., 2005).

The role of polyP metabolism in natural ecosystems has been supported by the results of a number of studies conducted exclusively in sedimentary environments. Gächter et al. (1988) observed that bacteria isolated from the sediments of the eutrophic Lake Sempach were able to bind and release P when exposed to alternating aerobic-anaerobic conditions. Similarly, Khoshmanesh et al. (1999) demonstrated that acetate amended wetlands sediment released a significant amount of the SRP, i.e. soluble reactive P, during anaerobic conditions after aerobic P enrichment. The most compelling evidence to date has been recently reported in a study by Schulz and Schulz (2005) with the giant marine sulfur bacterium *Thiomargarita namibiensis.* These authors were able to experimentally demonstrate that P released by these bacteria under anaerobic conditions in the presence of acetate can concentrate pore water phosphate high enough to drive spontaneous precipitation of phosphorus minerals. Such results explain the formation of major deposits of phosphorites in highly productive upwelling areas along the coast of Namibia, where the presence of a high biomass of this marine bacterium is commonly found.

Another line of evidence of the occurrence of polyP metabolism in nature has been provided by the presence of biogenic polyP in sediments revealed by the use of techniques such as Transmission Electron Microscopy (TEM) (Uhlmann and Bauer, 1988; Hupfer et al., 1995; Schulz and Schulz, 2005), and liquid state ^{31}P Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMR) (Hupfer et al., 1995, 2004; Carman et al., 2000; Khoshmanesh et al., 2002; Reitzel et al., 2006, 2007) as well as solid state ^{31}P-NMR (Sannigrahi and Ingall, 2005).

PolyP metabolism is apparently well spread in nature, and many microorganisms besides heterotrophs are capable of intracellular polyP storage. Different genera of phototrophs (e.g. Pick et al. 1990, Sudo et al. 1997, Eixler et al. 2006), including cyanobacteria (Barbiero and Welch 1992; Istvanovics et al. 1990), are able to store polyP in euphotic zones of aquatic environments. Settling of pelagic microorganisms might therefore introduce substantial amounts of polyP into the sediments.

c) Ecological significance of PolyP metabolism in P cycling

The retrieval of near-complete genomes of Accumulibacter from lab-scale EBPR enrichment culture using shotgun sequencing has allowed researchers to perform a comprehensive metabolic reconstruction of this organism (Garcia et al., 2006). These authors found multiple genes encoding functions more likely to be used in oligotrophic environments than in nutrient rich activated sludge. These include complete pathways for nitrogen and carbon fixation, high affinity phosphate transporters, and chemotaxis and flagellar genes.

Two recent studies using specific PCR primers have confirmed the presence of this organism in freshwater and associated sediments, and also, although rarely, in soils (Kunin et al., 2008; Peterson et al., 2008). Positive samples were collected at varying distances from WWTP performing EBPR (Peterson et al., 2008), suggesting that Accumulibacter is dispersed in the environment from the open activated sludge aeration basins enriched in this organism via aerosols. Kunin et al. (2008) propose that Accumulibacter populations are environmentally distributed as sparse high-density point sources, i.e. EBPR

sludges, which are linked via widespread diffuse aquatic reservoirs, thus constituting a metapopulation, i.e. a collection of contained populations connected by a small amount of gene flow (Hanski, 1999). Indeed, sludge samples obtained from two geographically remote laboratory-scale bioreactors with no known history of sludge transfer, one from Madison, Wisconsin, USA, and the other from Brisbane, Queensland, Australia, were dominated by highly similar strains, which shared over 95% nucleotide identity across most of the genome (Garcia et al. 2006), further supporting this theory.

The results presented in Chapter 2 provide further evidence of the presence of Accumulibacter in natural ecosystems. A significant aspect of this research is that the field site is located in Freeville, Tompkins County, NY, inserted in an agricultural landscape, far away from any WWTP, with no known history of soil amendments with activated sludge. Thus, contrarily to what Kunin et al. (2008) hypothesize about the origin of Accumulibacter in natural environments, this organism seem to naturally occur in this particular location and is, very likely, spread in natural ecosystems. These results also constitute the first report of spatial distribution patterns of Accumulibacter in soils, with the potential of relating such patterns to hydrological and pedological features in the landscape.

A puzzling piece of evidence, however, derives from the results described on Chapter 1. No polyP was detected in undisturbed soil cores maintained under fluctuating redox conditions in the presence of acetate, even though Accumulibacter was indeed detected in these cores. This suggests that other factors besides redox potentials and the availability of acetate might act as

regulators of polyP metabolism. Flanking microbial populations found in EBPR reactors have been proposed to provide nitrite for *Accumilibacter* anaerobic respiration (Garcia et al., 2006). Synthropic relations, i.e. when one species live on the products of another, seem therefore to be very important for the survival and predominance of Accumulibacter, explaining why it has not yet been possible to axenically cultivate this organism. Quorum sensing, on the other hand, is an important environmental monitoring system in which cell density and the associated secretion of specific signal compounds, commonly lactones or oligopeptides, coordinates the response of a bacterial population (Parsek and Greenberg, 2005; Schuster and Greenberg, 2007; Williams, 2007). It is possible that quorum sensing may somehow coordinate the ability of Accumulibacter to activate polyP metabolism and accumulate intracellular polyP once a threshold cell density has been reached. Further research is required, however, in order to test these theories.

The idea of microbial polyP as a temporal P sink has been controversially discussed, mostly because it is considered arguable by some authors that it actually accounts for significant P release, e.g. Golterman (2004). It is necessary to acknowledge, however, that microbial and chemical processes regulating P cycling are not mutually exclusive but in fact tightly coupled, observation sipported by Schulz and Schulz (2005). Thus, the ecological function of Accumulibacter and other unknown PAOs may potentially have a niche in the formation of P minerals though precipitation, rather than constituting a P sink in the microbial biomass itself. Such hypothesis remains by now only in speculations until further studies are conducted.

PolyP is a molecule involved in multiple biochemical processes that can result in an advantage for those microorganisms displaying this metabolism. The enzyme ppk1, responsible for intracellular polyP formation, has proven to be crucial for biofilm development (Rashid et al., 2000) and for providing protection against predation by environmental protozoa (Hall-Stoodley et al., 2004) and desiccation by UV light (Elasri and Miller, 1999), as well as for the development of phenotypic resistance to numerous stressors (Brown and Smith, 2003; Shiba et al., 1997). It is also involved in the general stress response (Hengge-Aronis, 2002), including the response to osmotic stress (Fraley et al., 2007; DoCampo et al., 2005). It is also involved in error-prone DNA replication (Stumpf and Foster, 2005), thus having a remarkable potential role in adaptive evolution.

Schulz and Schulz (2005) proposed that, in the case of *Thiomargarita namibiensis,* intracellular polyP breakdown is an auxiliary metabolism, from which this organism can gain the energy required for acetate uptake and storage in the absence of an electron acceptor. This explains why P release occurs only episodically and why P does not continuously accumulate with increased sediment depth. Such hypothesis is in accordance with the observation that the main function of the anaerobic phase on EBPR systems is to give PAOs a competitive advantage for substrate uptake over other heterotrophic bacteria subsequent growth and replication in the aerobic period.

2.2.3. Dissimilatory Fe reduction

a) Metabolic aspects of dissimilatory Fe reduction

Dissimilatory Fe reduction is a process that couples the oxidation of reduced organic and inorganic compounds to the reduction of Fe(III) in energy-conserving reactions that result in cell growth (Brock and Gustafson, 1976; Lovley et al., 1997). There is a wide diversity of bacteria and archaea with this metabolic capability (Lovley, 2000a,b). Nevertheless, environments where Fe reduction is an important electron acceptor process are dominated by the *Geobacteraceae* family (Anderson et al., 2003; Holmes et al., 2002; Roling et al., 2001; Snoeyenbos-West et al., 2000; Stein et al., 2001). *Geobacteraceae* belongs to the δ subclass of proteobacteria and comprises five genera, i.e. *Geobacter*, *Desulfuromonas*, *Desulfuromusa*, *Pelobacter* and *Malonomonas*, which have been isolated mostly from anoxic sedimentary environments (Holmes et al., 2004). All *Geobacteraceae*, with the exception of *Pelobacter*, are capable of complete oxidization of multicarbon compounds such acetate to carbon dioxide using Fe as electron acceptor (Lovley, 2000b). This group also show the capability of fixing nitrogen (Holmes et al., 2004, 2007), which likely allow these species to adapt and proliferate in nutrient limited environments.

Fe reducers display a variety of reduction strategies. They can reduce Fe either by direct contact between the organism and the oxide surface (Reguera et al., 2005), or by indirect mechanisms including outer-membrane cytochromes (Beliaev and Saffarini, 1998; Magnuson et al, 2001; Myers and Myers, 2001) and electron shuttles (Lovley et al., 1996). While this latter

strategy is probably effective under certain circumstances, it is energetically expensive, with a cost that would quickly become a net energy loss for the organism if is used on regular basis. Thus, the fact that *Geobacteraceae* species need to contact Fe oxides in order to reduce them might be energetically favorable given the direct contact with the substrate.

Besides acetate, common electron donors for dissimilatory Fe reduction are short-chain carboxylic acids (Lovley and Phillips, 1988; Lovley, 1995a,b), alcohols and aromatic compounds (Lovley et al. 1993; Nealson and Saffarini, 1994), and glucose (Lovley and Coates, 2000). Evidence suggests, however, that glycolysis intermediates and not glucose directly might be responsible for transferring electrons to Fe oxides (Lovley, 2000a).

The nature of the available oxidized Fe forms as substrate for reduction can have a strong influence in the process rates, being amorphous oxides in general more rapidly reduced than crystalline forms given their higher specific surface area (Langenhoff et al., 1997; Thamdrup, 2000).

b) Evidence of dissimilatory Fe reduction in natural ecosystems

Early models of Fe reduction emphasized the abiotic aspects of this process (Einsele, 1936, 1938; Mortimer, 1941, 1942). Such view was challenged by the discovery of dissimilatory Fe reduction, which has been proposed to be the main mechanism for Fe reduction in sedimentary environments (Lovley and Phillips, 1988; Lovley, 1995a,b).

A number of Fe reducers have been isolated from a wide range of environments including oil field fluids (Semple and Westlake 1987), pristine deep aquifers (Lovley et al., 1990), estuarine sediments (Caccavo et al., 1992), petroleum contaminated soils (Caccavo et al., 1994), and thermal hot springs (Slobodkin et al., 1997). The availability of enrichment cultures has allowed a closer study of this metabolic process.

The structure of microbial communities of Fe reducers has been addressed though the phylogenetic analysis of 16S rRNA sequences retrieved from environmental samples and the use of probes targeted to specific 16S rRNA regions (Olsen et al., 1986; Pace et al., 1986; Giovannoni et al., 1987; Stahl et al., 1988; DeLong et al., 1989; Eden et al., 1991; Ward et al., 1992; Amann et al., 1995).

More recently, the metabolic state of *Geobacteraceae* in subsurface environments has been inferred based on the *in situ* expression of mRNA of the α subunit of the dinitrogenase (nifD) genes, protein responsible for nitrogen fixation. The addition of acetate to subsurface sediments poor in organic matter and nitrogen stimulated the growth of *Geobacteraceae* and Fe(III) reduction, as well as the expression of the target gene (Holmes et al., 2004). A similar study was conducted based on the mRNA levels of the citrate synthase (gltA) gene, a key enzyme in the central metabolism of *Geobacteraceae.* mRNA expression in two groundwater sources were correlated to the levels of acetate present in the samples (Holmes et al., 2005).

The presence of *Geobacteraceae* was confirmed in the VFS in the study site by the results reported in Chapter 2. There were important issues regarding primers specificity, since the primer set used targeted also a number of other sequences not belonging to this particular group. The information obtained with qPCR, however, is still useful in terms of a relative comparison between the samples, since all of them including the soil cores were taken from the same filed site, and thus display the same variation. It is worth to notice that in contrast to Accumulibacter, *Geobacteraceae* was only found in some of the samples analyzed. As a result, the spatial distribution patterns revealed *Geobacteraceae* is clustered at the entrance of one of the VFS cells, which according to field observations is the wettest location in the site, thus resembling subsurface environments where *Geobacteraceae* has been normally found.

c) Ecological significance of dissimilatory Fe reduction in P cycling

Dissimilatory Fe reduction rates, and hence P cycling coupled to this process, are in general difficult to estimate because they are influenced by a number of factors. The availability of amorphous Fe oxides as substrate is one of them, since they are more rapidly reduced than crystalline forms given their higher specific surface area (Langenhoff et al., 1997; Thamdrup, 2000). In soils and sediments, the content of Fe hydroxides ranges from 0.2 to 20% (Scheffer and Schachtschabel, 1998). Thus, besides CO_2, they potentially represent the most important electron acceptor in many anoxic environments (Dominik et al., 2004).

Another important factor is that reduced Fe is rapidly oxidized by molecular oxygen, forming insoluble Fe hydroxide ($Fe(OH)_3$), hematite (Fe_20_3), goethite (FeOOH) and other oxides, reaction that apparently outcompetes biological oxidation (Nealson and Saffarini, 1994). Fe is, therefore, capable to recycle as many as 100-300 times, as determined in offshore sediments (Canfield et al., 1993). This process could also be very important in soils with fluctuating water tables, such as VFSs and other HSAs, which can expose recently reduced Fe to oxygen, thus favoring the formation of fresh Fe oxides.

Sulfide can interact strongly with Fe, forming insoluble iron monosulfides (FeS), which can be further reduced by elemental sulfur to the more thermodynamically stable pyrite (FeS_2) at high sulfide concentrations (Howarth, 1979). Sulfate (SO_4^{2-}) reduction into sulfide is an important process in the cycling of organic matter in coastal marine environments (Jørgensen, 1982), where SO_4^{2-} concentrations are usually high. Its importance in soils is in general more limited given their low SO_4^{2-} content (Nealson and Saffarini, 1994).

Fe reduction rates also rely on the availability of a preferential C source. Many Fe reducers, including *Geobacteraceae,* use acetate preferentially as a substrate over other electron donors (Lovley, 2000b). Acetate has been identified as the main intermediate in anaerobic mineralization of organic carbon (C) in many aquatic ecosystems (Lovley and Klug, 1982; Rothfuss and Conrad, 1993), playing also an important role in upland environments, such as prairie and forest soils, where anaerobic conditions are restricted to microsites in soil aggregates (Küsel and Drake, 1994; Wagner et al., 1996). In rice field

soils, acetate is the dominant fatty acid, often accumulating at high concentrations within two weeks after flooding (Inubushi et al., 1984; Klüber and Conrad, 1998; Krylova et al., 1997; Sugimoto and Wada, 1993). Glucose apparently served as an important precursor of acetate in such environments, accounting for 54 to 81% of the acetate produced (Chidtaisong et al., 1999), being also the most abundant monosaccharide in field rice soils as well as in freshwater and marine ecosystems (King and Klug, 1982; Wicks et al., 1991.Jørgensen and Jensen, 1994; Boschker et al., 1995; Dulov et al., 1995; Hanisch et al., 1996).

The presence of *Geobacteraecae* in the field site is in accordance with observed Fe reduction in the soil cores containing glucose during flooding, as described in Chapter 1. Surprisingly, no Fe reduction was observed in the cores containing acetate. It is speculated that initial concentrations of acetate favored other microbial groups instead during the early stages of soil reduction during flooding, while in the cores containing glucose was in fact the acetate formed from glucose the electron donor used for Fe reduction.

An intriguing aspect of the results is that no concomitant P release was observed during Fe reduction. Based on the available information is not possible to determine if whether P_i was indeed released along with Fe and rapidly consumed, or if it was not released at all. This latter implies the lack of P bound to amorphous Fe oxides in the study site, which seems unlikely. Even when the content of amorphous Fe oxides in the study site is unknown, the occurrence of Fe reduction indicates the presence of at least a fraction of them. In addition, the clone libraries described in the same chapter revealed

the presence of sequences of other Fe reducer species, e.g. *Anaeromyxobacter* and *Ferribacterium limneticum*, which similarly to *Geobacteraceae* also exhibit the ability to oxidize acetate, suggests that Fe reduction might be an important process in the field site and cannot be exclusively attributed to *Geobacteraceae.*

2.3. VFSs and NPS pollution

The implementation of buffer zones is a widely used strategy for controlling NPS pollution of P. They are planned to reduce the volume of surface and subsurface flow, with the main purpose of intercepting, retaining, and transforming nutrients and pollutants, as summarized by Dorioz et al. (2006). VFSs are considered most effective in removing sediment bound P (Abu-Zreig et al., 2003), with a more limited effectiveness, however, for decreasing dissolved P (DP) (Schmitt et al., 1999; Sharpley et al., 2006).

2.3.1. The P buffer effect of VFSs

The buffer effect of VFSs relies primarily in two major processes: deposition and infiltration.

The presence of a continuous vegetation cover provides a greater resistance to surface flow, which results in a decrease in the transport capacity for solid material, where the excess particles progressively sedimented and trapped (Munos-Carpena et al., 1999). This deposition is usually greater in the front edge of the grass buffer strip, with most of the sediment (53-86% of the input

load) being retained within the first 5 m (Dillaha et al., 1989; Magette et al. 1989). It is also accompanied by a granulometric sorting, first the coarser fractions, then the finer ones. The trapping of smaller particles, such as clays, requires a kind of deposition that Dorioz et al (2006) defined as turbulent filtration, which occurs when runoff flows though the leafy matrix of the grass and herb covering the buffer zone, being especially prevalent further down the VFS. In many cases, only the coarse particles are retained (Hayes et al., 1984; Robinson et al., 1996), while the fraction of P bound to the finest particles is not (Uusi-Kämppä et al., 1997).

The runoff water reaching a VFS flows over a porous surface with continuous vegetation cover that provides a great resistance to surface flow and thus slows it down. At the same time, a dense root system increases the permeability of the surface soil layers (Magette et al., 1989; Rose et al., 2003), thus favoring the water infiltration in the soil. This process is responsible for the retention of the dissolved P forms (Dillaha et al., 1986). As DP is actively fixed by the soil constituents, the displacement of P to depth is limited to the surface layers. P retention in this case is therefore controlled by these basic physico-chemical mechanisms. Once this P is in the soil, the potential for later reactions remain, such as absorption by plants, adsorption onto the soil, or chemical precipitation.

2.3.2. Effectiveness of VFSs

Based on the analysis of 11 different studies, Dorioz et al. (2006) reported that VFSs are able to limit significantly the transfer of sediment to surface waters.

Retention ranges from 40 to 100%, with a reduction higher than 50% in more than 95% of the cases. They are seldom 100% effective, since clays, often heavily loaded with P, are generally only weakly retained. The situation is rather different for DP, whose retention commonly varies between 20 and 30%, ranging from (-)83 to (+)95%, The negative values indicate that the load of DP increases across the VFS (Dillaha et al., 1989; Uusi-Kämppä et al., 2000; Trévisan and Dorioz, 2001; Duchemin and Madjoub, 2004).

Given its impact in water infiltration, soil texture may largely affect P retention, particularly dissolved P forms. Schwer and Clausen (1989) reported a large difference in retention of TP and DP between two VFSs, one established on a sandy soil (92 and 98% retention, respectively) and the other on silty clay (33 and 12%, respectively). A higher retention capacity on sandy soils was also observed by Magette et al. (1989). Such differences, however, are somehow counterintuitive considering the higher P sorption capacity of clays compared to sand.

The accumulation of sediment in VFSs could result in the release of dissolved P during subsequent runoff periods (Uusi-Kämppä et al., 2000). In many cases, this is only a temporary degradation of the system function, since after flooding with runoff water, infiltration and evaporation allow vegetation to regrow through most of the sediment deposits, thus contributing to the stabilization of the stored particles and restoring the stability and the retention capacity of the system (Parmeland, 1995). Nevertheless, in the long term, such stabilization and restoration become insufficient due to the continuous accumulation of sediment. Soil microtopography then changes, diminishing the

permeability of the system and thus favoring internal erosion (Dillaha and Inamdar, 1997). Similarly, the accumulation of TP, which mostly occurs in the top 2 - 3 cm of VFSs soils (Dorioz et al., 2006), can also saturate the system with P thus generating conditions that favor DP release (Muscutt et al., 1993).

As a VFS is gradually saturated with both sediment and different P forms, it becomes particularly vulnerable to flooding, with the time span of the flooding being positively correlated to the degradation level of the system. As mentioned above, flooding can impose an osmotic shock on soil microbes and generate a shift in the microbial community, thus resulting in enhanced P release dominated by organic P forms derived from microbial decay. In addition, upon the depletion of oxygen in flooded soils, anaerobic degradation of organic matter takes place with a succession of thermodynamically favored reactions starting with the reduction of nitrate (NO_3^-), followed by Mn(IV), Fe(III), sulfate (SO_4^{2-}) and carbon dioxide (CO_2), in this order as each electron acceptor is successively depleted that proceed as each electron acceptor is successively depleted (Fig. 3.1). Acetate is the main intermediate in anaerobic mineralization of organic carbon in many sediments and soils, and could certainly promote polyP metabolism by PAOs as well as dissimilatory Fe reduction in reducing soil conditions. A VFS vulnerable to flooding is also prone to produce runoff, which can superficially transport P and other nutrients released to water bodies.

3. A conceptual framework for soil P dynamics

This paper summarizes the existing evidence and theories about the different process regulating soil P dynamics. Many of the cases discussed here are presented to generalize the major aspects of particular process, but exceptions to such generalizations are acknowledged.

The processes described here can divide in those affecting P retention and those involved in P release. Both categories depend on the soil redox state, i.e. P retention takes place under oxidizing conditions and P release under reducing conditions. In an oxidizing environment, soil P retention takes place. The most significant physical-chemical process involved appears to be P sorption to Fe. Fe oxides constitute a major temporal sink, which can subsequently be reduced and thus participate in P release. While Mn and Al oxides can also account of some P retained in soils, Mn effects are limited to early stages of soil reduction and to the presence of a large content of reactive Mn oxides. Precipitation is another important physical-chemical process controlling P retention. In acid soils, P retention is generally controlled by the solubility of variscite ($AlPO_4 \cdot 2H_2O$), while in non acid soils the precipitation of different forms of Ca phosphate plays a more important function. In terms of microbial processes, P_i uptake by PAOs to store intracellular polyP might also be an important mechanism for P retention in soils.

As soil flooding proceeds, the environment turns gradually anaerobic (Fig. 3.1). In such conditions, the soils increasingly become more vulnerable to P release as the result of a sequence of microbially mediated processes. P

release dominated by organic forms immediately follows rewetting, as the result of the decay of the soil microbial biomass due to an initial osmotic shock. Later on, decay continues due to a shift in the soil microbial community caused by the change in the prevailing environmental conditions.

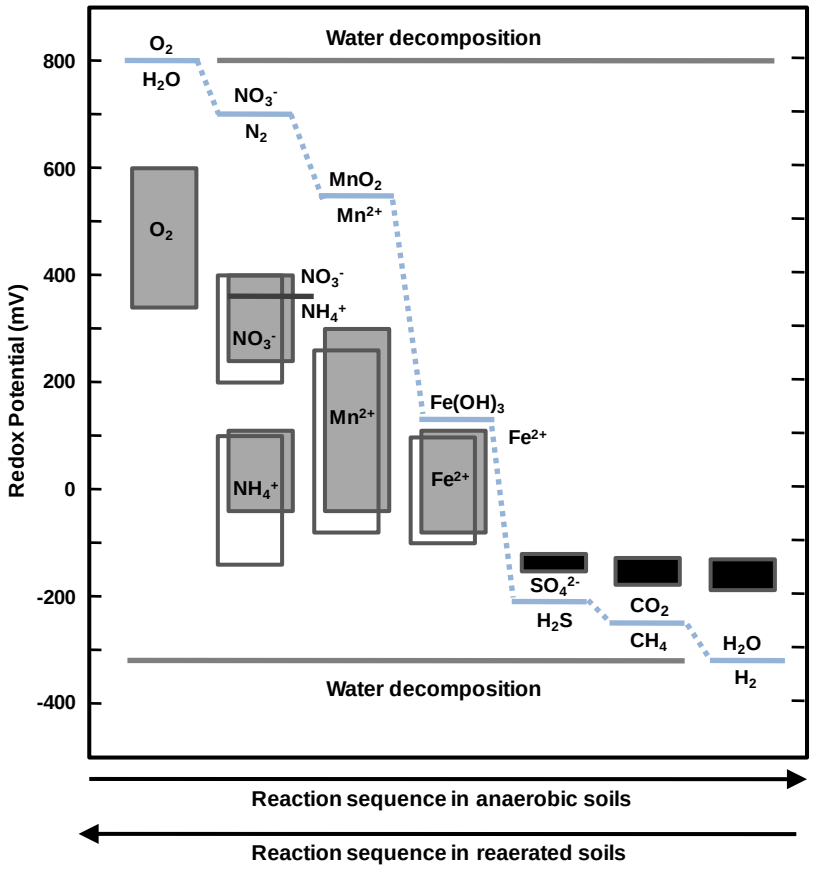

Figure 3.1. Reduction and oxidation sequence in soils at pH 7.0. Theoretical potentials are indicated by solid lines, assuming equal activities of reduced and oxidized species. The pressure of H_2 is arbitrarily set at 10^{-3} atmosphere. Measured ranges of soil potentials over which the indicated species react during soil reduction and oxidation are specified by boxes: shaded for reduction, open for oxidation and black for initial appearance of the reduced form during reduction. (Source: modified from McBride, 1994).

The ecological function of PAOs, this time releasing Pi in order to take acetate, might potentially also contribute to P fluxes from flooded soils. It might also have a niche in the formation of P minerals though precipitation by increasing Pi concentrations in pore water. This hypothesis, as well as the role of PAOs itself in soil P dynamics, remains for now only in speculations. The reduction of Fe(III) oxides is primarily the result of dissimilatory Fe reduction, since Fe reduction by sulfide is not expected to be significant in soils given the low content of sulfate. This results in the concomitant release of reduced Fe and P_i.

When precipitation occurs over flooded soils, a fraction of P and other nutrients released from soils can be transported by surface runoff, depending on the rainfall intensity. The remaining fraction remains in the system. When subsequent infiltration and evaporation take place, soil structure recovers and the soils gradually become aerobic. Some of the reduced Fe(II) can be then rapidly oxidized and form fresh Fe(III) oxides. Increasing aerobic conditions can also promote the development of PAOs populations in the soil, which can, after have taken up acetate as substrate, grow and replicate in this increasingly aerobic environment.

It is possible then, based on the evidence presented in this section, to summarize a conceptual framework of soil P dynamics, which is presented in Fig. 3.2.

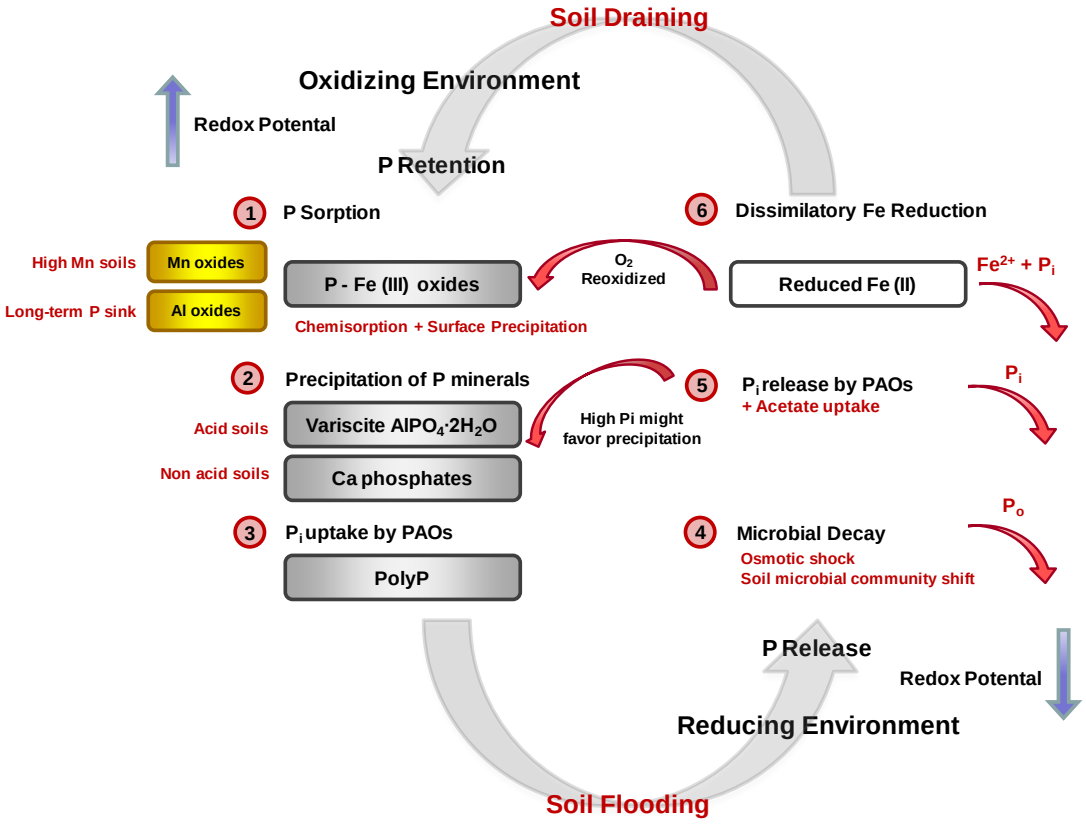

Figure 3.2. Conceptual framework for soil P dynamics. The soild boxes represent the pools of different compounds that can potentially result in short and long term P sinks. The empty boxes represent pools of compounds temporally depleted as the result of a particular process, such as Fe reduction. Oxidizing and reducing environments are presented as a cycle of soil draining and flooding, respectively, with the resulting changes in the redox potentials.

4. Future research

Based on the evidence and many of the aspects of soil P dynamics discussed in this paper, it is possible to visualize future lines of research.

The results reported in Chapter 2 constitute significant evidence of the occurrence of Accumulibacter in natural environments. Further studies using molecular techniques conducted in different ecosystems, including minimally impacted soils and water bodies, as well as environments exposed to the effects of human activities, might provide additional clues about the global distribution of Accumulibacter, as well as insights of its potential ecological functions.

The use of qPCR in combination with reverse transcriptase PCR (RT-PCR) can be a powerful tool to quantitatively estimate *in situ* the activity of Accumulibacter and other PAOs, as well as *Geobacteraceae.* It can also offer valuable information about the metabolic status of these communities in soils and natural environments.

The development of experiments using porous media to study biofilm formation by Accumulibacter as well as aspects of dynamic P transport could significantly contribute to gain a better understanding of the mechanisms related to soil P dynamics by controlling the physical–chemical properties of the media, thus minimizing the interference of factors inherent to soil heterogeneity. In addition, this experimental design could also be used to test the effects of Ca precipitation under low and high Ca concentrations in order to

establish a link between polyP metabolism and precipitation of P minerals in soils.

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Table A1. Coefficient of Determination (r^2) for the duplicates of the soil cores experiment (Chapter I). $r^2 \geq 0.7$ in red.

Cycle	Cores	pH	DO mg/L	Redox mV	Acetate mg/L	TP mg/L	P_i mg/L	P_o mg/L	Ca mg/L	NO_3^- mg/L	Mn mg/L	Fe mg/L	SO_4^{2-} mg/L
1	1 and 2	0.70	0.76	0.86	0.75	0.90	0.01	0.93	0.97	0.79	0.42		0.92
1	3 and 4	0.53	0.82	0.96	0.81	0.56	0.01	0.87	0.99	0.94	0.75	0.39	0.82
1	5 and 6	0.61	0.98	0.97	0.71	0.87	0.62	0.86	0.95	0.88	0.71	0.35	0.12
2	1 and 2	0.84	0.80	0.76	0.35	0.86	0.37	0.89	0.69	0.47	0.81		0.04
2	3 and 4	0.93	0.95	0.95	0.88	0.87	0.00	0.92	0.90	0.12	0.33		0.22
2	5 and 6	0.95	0.96	0.94	0.96	0.90	0.92	0.91	0.96	0.62	0.34	0.50	0.97
3	1 and 2	0.75	0.97	0.76	0.63	0.97	0.64	0.97	0.96	0.63			0.77
3	3 and 4	0.75	0.98	0.87	0.92	0.95	0.95	0.96	0.89	0.51	0.92		0.55
3	5 and 6	0.87	0.97	0.97	0.78	0.98	0.96	0.97	0.97	0.64	0.56	0.31	0.94
PE	1 and 2	0.83	0.05	0.87		0.78	0.96	0.40	0.77	0.04		0.23	0.94
PE	3 and 4	0.96	0.73	0.97		0.94	0.97	0.65	0.44	0.05		0.32	1.00
PE	5 and 6	0.97	0.57	0.82		0.96	0.97	0.49	0.48	0.03		0.32	1.00

PE: P Enrichment Period

APPENDIX II

Table B1. Average and standard deviations (SD) for qPCR results. The average for some samples is represented by a single value, therefore no SD is reported in those cases.

Sample		Accumulibacter		*Geobacteraceae*		Total bacteria	
		Average	SD	Average	SD	Average	SD
Field sample	**1**	1.40.E+03	1.22E+03	1.32E+02	9.33E+01	8.71.E+05	4.27E+05
Field sample	**2**	4.39.E+03	-	4.80E+02	1.56E+02	1.38.E+06	4.35E+05
Field sample	**3**	3.27.E+02	3.00E+02	2.47E+03	-	3.56.E+05	2.26E+05
Field sample	4	9.56.E+02	6.67E+02	7.44E+01	9.24E+01	1.08.E+06	8.78E+05
Field sample	5	3.39.E+03	1.04E+03	6.03E+01	1.88E+01	1.35.E+06	6.64E+05
Field sample	6	1.52.E+03	5.76E+02	4.51E+01	3.13E+01	3.09.E+05	8.39E+04
Field sample	7	1.16.E+03	6.08E+02	1.05E+02	-	5.49.E+05	1.53E+05
Field sample	8	2.86.E+03	1.04E+03	0.00E+00	-	1.66.E+06	1.08E+06
Field sample	9	2.47.E+03	7.69E+02	0.00E+00	-	9.54.E+05	2.95E+05
Field sample	10	2.71.E+03	7.44E+02	1.28E+02	3.56E+01	1.18.E+06	6.79E+05
Field sample	11	2.04.E+02	1.39E+02	0.00E+00	-	1.11.E+05	6.17E+04
Field sample	12	9.23.E+02	1.17E+02	0.00E+00	-	7.00.E+05	3.22E+05
Field sample	13	3.11.E+03	3.30E+03	0.00E+00	-	4.28.E+05	1.19E+05
Field sample	14	1.83.E+02	1.20E+02	4.88E+01	1.62E+01	4.96.E+05	1.03E+05
Field sample	15	9.13.E+02	8.65E-01	1.87E+02	-	1.33.E+06	4.70E+05
Field sample	16	4.32.E+03	2.68E+03	1.82E+02	1.56E+02	1.16.E+06	2.95E+05
Field sample	17	4.30.E+02	3.73E+02	0.00E+00	-	9.03.E+05	2.48E+05
Field sample	18	6.35.E+02	9.31E+01	0.00E+00	-	1.21.E+06	5.81E+04
Field sample	19	2.41.E+03	5.10E+01	0.00E+00	-	1.58.E+06	2.88E+05
Field sample	20	1.31.E+03	1.01E+03	4.38E+01	2.94E+01	2.82.E+06	4.93E+05
Field sample	21	5.26.E+02	3.65E+02	0.00E+00	-	2.71.E+05	4.04E+04
Field sample	22	8.26.E+02	5.87E+02	0.00E+00	-	4.40.E+05	3.64E+04
Field sample	23	4.69.E+02	5.90E+01	0.00E+00	-	1.81.E+05	3.97E+04
Field sample	24	7.76.E+02	4.68E+02	1.26E+02	-	2.82.E+05	3.77E+03
Field sample	25	3.15.E+03	9.06E+02	1.11E+02	3.06E+01	7.36.E+05	1.16E+05
Field sample	26	3.51.E+02	4.13E+02	0.00E+00	-	1.12.E+05	3.91E+04
Field sample	27	1.54.E+03	4.59E+02	0.00E+00	-	3.78.E+05	1.50E+05
Field sample	28	9.79.E+02	4.77E+02	4.67E+01	-	5.86.E+05	8.56E+04
Field sample	29	8.93.E+02	5.67E+02	8.54E+01	5.56E+01	9.34.E+05	4.50E+05
Field sample	30	1.29.E+03	2.18E+02	2.74E+02	1.07E+02	7.25.E+05	2.72E+05
Field sample	31	3.00.E+03	5.99E+02	5.80E+01	1.94E+00	2.36.E+06	8.30E+05
Field sample	32	6.50.E+02	3.41E+02	7.17E+01	2.73E+01	1.35.E+06	4.51E+05

Sample		Accumulibacter		*Geobacteraceae*		Total bacteria	
		Average	SD	Average	SD	Average	SD
Field sample	33	1.83.E+03	3.84E+02	0.00E+00	-	1.40.E+06	1.75E+05
Field sample	34	9.96.E+02	3.71E+02	0.00E+00	-	7.44.E+05	4.62E+05
Field sample	35	5.16.E+02	6.45E+01	5.95E+01	3.92E+01	1.75.E+06	1.22E+06
Field sample	36	5.95.E+02	5.91E+02	9.61E+01	1.03E+02	3.78.E+05	3.23E+04
Field sample	37	1.88.E+02	2.51E+02	4.04E+01	4.57E+00	1.79.E+06	1.38E+05
Field sample	38	4.21.E+02	2.45E+02	0.00E+00	-	1.06.E+06	2.59E+05
Field sample	39	6.81.E+02	3.07E+02	0.00E+00	-	1.98.E+06	1.32E+06
Field sample	40	2.97.E+02	1.69E+02	8.14E+01	5.56E+01	7.02.E+05	9.12E+04
Field sample	41	6.47.E+02	6.46E+02	4.55E+01	-	6.71.E+05	6.97E+04
Field sample	42	8.30.E+02	5.59E+02	0.00E+00	-	1.50.E+06	3.59E+05
Field sample	43	1.25.E+03	1.09E+03	9.25E+01	-	5.00.E+05	1.74E+05
Field sample	44	3.42.E+02	6.37E+01	0.00E+00	-	1.24.E+06	2.94E+05
Field sample	45	4.08.E+02	-	0.00E+00	-	1.99.E+06	7.52E+05
Field sample	46	1.15.E+03	4.87E+02	0.00E+00	-	3.15.E+06	6.68E+05
Field sample	47	5.04.E+03	2.81E+03	0.00E+00	-	1.85.E+06	9.40E+05
Field sample	48	7.34.E+02	5.71E+02	0.00E+00	-	1.05.E+06	1.74E+05
Field sample	49	3.21.E+02	2.28E+02	6.12E+01	7.86E+01	6.19.E+05	1.21E+05
Field sample	50	8.13.E+02	3.98E+02	1.97E+03	2.76E+03	1.58.E+06	2.69E+05
Field sample	51	6.61.E+02	5.76E+01	0.00E+00	-	8.63.E+05	1.42E+05
Field sample	52	4.80.E+02	2.84E+02	3.38E+01	2.50E+01	6.71.E+05	2.21E+05
Field sample	53	8.48.E+02	5.27E+02	6.50E+01	2.25E+01	8.25.E+05	1.74E+05
Field sample	54	5.08.E+02	3.90E+02	1.41E+02	2.32E+01	9.18.E+05	7.80E+05
Field sample	55	9.44.E+02	4.84E+02	0.00E+00	-	2.75.E+06	1.54E+05
Field sample	56	4.61.E+02	1.25E+02	0.00E+00	-	6.11.E+05	6.65E+04
Field sample	57	3.54.E+02	1.12E+02	0.00E+00	-	1.55.E+05	4.15E+04
Field sample	58	1.49.E+02	3.39E+01	0.00E+00	-	4.32.E+05	4.98E+04
Field sample	59	1.55.E+02	-	0.00E+00	-	3.80.E+05	1.91E+05
Field sample	60	3.47.E+02	2.83E+01	0.00E+00	-	1.12.E+06	5.85E+05
Field sample	61	4.01.E+02	1.47E+02	0.00E+00	-	1.50.E+06	5.35E+05
Field sample	62	3.33.E+02	6.99E+01	0.00E+00	-	5.84.E+05	1.47E+05
Field sample	63	1.79.E+03	2.34E+02	0.00E+00	-	1.47.E+06	3.68E+05
Field sample	64	1.39.E+03	1.23E+03	3.91E+02	2.84E+02	1.29.E+06	1.23E+05
Field sample	65	7.94.E+02	6.84E+02	7.20E+01	1.81E+00	9.81.E+05	2.41E+05
Field sample	66	1.80.E+02	1.80E+01	0.00E+00	-	4.89.E+05	2.52E+05
Field sample	67	3.13.E+03	2.57E+03	1.00E+00	-	3.93.E+05	1.24E+05
Leachate sample	P1	2.40.E+02	1.28E+01	6.48E+01	-	4.62.E+06	4.21E+06
Leachate sample	P2	4.45.E+03	3.09E+03	0.00E+00	-	1.31.E+07	3.47E+06
Leachate sample	P3	3.20.E+02	3.39E+02	3.05E+01	9.62E+00	3.33.E+06	1.15E+06
Leachate sample	P4	4.30.E+02	4.31E+02	0.00E+00	-	2.22.E+06	6.70E+05
Core 1	T0	0.00.E+00	0.00E+00	0.00E+00	-	1.99.E+06	1.53E+06

		Accumulibacter		*Geobacteraceae*		Total bacteria	
Sample		Average	SD	Average	SD	Sample	Average
Core 2	T0	3.48.E+01	6.03E+01	1.04E+01	-	1.84.E+06	1.29E+05
Core 3	T0	0.00.E+00	0.00E+00	0.00E+00	-	1.01.E+06	2.49E+05
Core 4	T0	0.00.E+00	0.00E+00	0.00E+00	0.00E+00	0.00.E+00	0.00E+00
Core 5	T0	2.08.E+01	3.60E+01	0.00E+00	-	1.05.E+06	1.23E+06
Core 6	T0	0.00.E+00	0.00E+00	0.00E+00	-	0.00.E+00	0.00E+00
Core 1	Cycle 1	6.04.E+02	8.14E+02	0.00E+00	-	5.87.E+05	5.21E+04
Core 2	Cycle 1	0.00.E+00	0.00E+00	0.00E+00	-	1.04.E+06	6.19E+05
Core 3	Cycle 1	6.05.E+01	5.33E+01	0.00E+00	-	1.95.E+06	8.81E+05
Core 4	Cycle 1	5.24.E+01	9.08E+01	0.00E+00	-	2.08.E+06	3.34E+05
Core 5	Cycle 1	0.00.E+00	0.00E+00	0.00E+00	-	1.68.E+06	1.27E+05
Core 6	Cycle 1	1.80.E+02	6.74E+01	0.00E+00	-	1.31.E+06	1.29E+06
Core 1	Cycle 2	0.00.E+00	0.00E+00	9.13E+00	1.27E+01	1.12.E+06	1.46E+06
Core 2	Cycle 2	0.00.E+00	0.00E+00	0.00E+00	-	1.07.E+06	1.45E+06
Core 3	Cycle 2	2.08.E+01	3.61E+01	0.00E+00	-	0.00.E+00	0.00E+00
Core 4	Cycle 2	1.68.E+02	6.96E+01	0.00E+00	-	1.16.E+06	3.13E+04
Core 5	Cycle 2	1.84.E+02	2.03E+02	0.00E+00	-	1.51.E+06	3.43E+05
Core 6	Cycle 2	0.00.E+00	0.00E+00	0.00E+00	0.00E+00	0.00.E+00	0.00E+00
Core 1	Cycle 3	5.78.E+01	8.18E+01	0.00E+00	-	1.46.E+06	8.93E+04
Core 2	Cycle 3	1.87.E+02	6.40E+01	1.71E+02	5.52E+01	4.82.E+05	6.68E+05
Core 3	Cycle 3	9.68.E+02	9.45E+02	6.38E+01	-	1.19.E+06	4.66E+05
Core 4	Cycle 3	0.00.E+00	0.00E+00	3.68E+01	2.89E+01	3.55.E+05	2.78E+05
Core 5	Cycle 3	1.20.E+02	2.98E+01	5.36E+01	3.75E+01	6.78.E+05	2.65E+05
Core 6	Cycle 3	2.39.E+03	3.71E+02	2.72E+02	3.73E+01	1.31.E+06	4.69E+05
Core 1	PE	4.04.E+02	1.02E+02	1.29E+02	6.58E+01	1.99.E+06	5.02E+05
Core 2	PE	5.52.E+01	7.81E+01	1.75E+02	3.12E+00	1.87.E+06	6.01E+05
Core 3	PE	0.00.E+00	0.00E+00	0.00E+00	0.00E+00	0.00.E+00	0.00E+00
Core 4	PE	0.00.E+00	0.00E+00	0.00E+00	0.00E+00	1.13.E+00	1.95E+00
Core 5	PE	0.00.E+00	0.00E+00	0.00E+00	0.00E+00	0.00.E+00	0.00E+00
Core 6	PE	5.66.E+01	9.80E+01	0.00E+00	-	2.32.E+06	2.86E+05
Blank		2.78.E+02	-	0.00E+00	-	3.26.E+03	9.90E+02